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Clinical and Experimental

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VOL. 46 SEPTEMBER, 1955 NO. 3

CLINICAL AND EXPERIMENTAL

RENAL ELECTROLYTE TRANSPORT IN NORMAL AND NEPHROTIC CHILDREN

EFFECTS OF SIMULTANEOUS INFUSION OF CARBONIC ANHYDRASE INHIBITOR AND NONREABSORBABLE ANION

J. METCOFF, M.S., M.D., J. A. JAMES, M.B., M.R.C.P.,* G. GORDILLO, M.D.,** AND I. ANTONOWICZ, M.S.
BOSTON, MASS.

IN A previous study[1] sodium plus nonreabsorbable anion loads were infused in groups of normal and nephrotic children. In the normal subjects, excretion of the load was covered by an equivalent quantity of fixed cation, mainly sodium. The principal results in the nephrotic children were as follows: (1) Excretion of sodium was impaired during the edematous phase, but returned to normal following diuresis. (2) Excessive excretion of potassium, usually in amounts greater than those simultaneously filtered, was demonstrated both before and after diuresis.

These results showed that the excessive excretion of potassium, typical of the nephrotic syndrome, was not dependent directly upon increased sodium reabsorption, and was perhaps related to a defect in the tubular mechanisms governing potassium excretion.

According to the hypothesis of Pitts,[2] modified by Berliner,[3] tubular potassium excretion is governed by an ion-exchange mechanism, potassium

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TABLE I. AVERAGED

PATIENT* HT. WT.	AGE (YR.) SA† (M^2)	PERIOD‡	TIME (MIN.)	PERIODS (NO.)	CLEARANCE INULIN ML./MIN. /M^2	CLEARANCE PAH	SERUM§ pH	SERUM§ CO_2 MM/L.	SERUM§ Na MM/L.	SERUM§ K MM/L.
Control Subjects.—										
R. L.	5 2/12	Control	30-75	3	69	335	7.42	26.1	133	3.2
101 cm.	0.65	Diamox	75-135	4	53	275	7.33	26.4	142	2.8
15.43 kg.		Diamox + PAH	135-210	4	49	—	7.33	23.8	133	2.4
D. C.	7	Control	30-68	3	62	370	7.32	27.4	123	4.0
106 cm.	0.7	Diamox	68-128	4	39	207	7.33	26.6	117	3.6
17.0 kg.		Diamox + PAH	128-213	4	33	—	7.37	23.7	117	3.1
R. A.	6 1/12	Control	50-95	3	52	263	7.45	26.6	131	4.0
106 cm.	0.70	Diamox	95-140	3	35	197	7.43	26.1	133	3.2
16.88 kg.		Diamox + PAH	167-242	4	34	—	7.42	23.7	131	2.7
Nephrotic Patients.—										
M. N.	2 10/12	Control	30-75	3	54	298	7.46	23.2	128	3.7
96 cm.	0.65	Diamox	90-136	4	51	238	7.47	24.2	124	4.0
16.30 kg.		Diamox + PAH	136-225	5	42	—	7.45	23.7	119	2.9
S. W.	6 6/12	Control	30-75	3	20	213	7.34	21.5	123	4.6
114 cm.	0.85	Diamox	75-135	4	22	250	7.42	21.9	125	4.1
25.00 kg.		Diamox + PAH	135-211	4	20	—	7.43	21.1	127	3.8
L. W.	6 5/12	Control	31-91	3	18	312	7.36	18.5	136	4.2
119.5 cm.	0.84	Diamox	91-196	5	22	364	7.34	17.9	136	4.0
35.12 kg.		Diamox + PAH	196-286	4	20	—	7.33	18.5	139	3.6
E. M.	5 2/12	Control	30-70‖	2	25	213	7.28	21.4	136	4.7
104.2 cm.	0.67	Diamox	120-160	2	26	290	7.30	21.2	133	3.2
20.1 kg.		Diamox + PAH	210-270	3	46	—	7.22	20.9	130	3.2

*All patients were females.

†Surface area.

‡Data for preinfusion collection periods and initial equilibration periods were omitted since not pertinent.

§Sodium and potassium values corrected for binding factors (serum values × 0.95).

‖Results of incomplete urine collections in intervening periods were discarded.

and hydrogen ions in the tubule cell competing for excretion in exchange for sodium within the lumen. Carbonic anhydrase inhibitors, by blocking hydration of carbon dioxide, inhibit hydrogen-ion production and therefore favor excretion of potassium, together with sodium and bicarbonate. Urinary changes consistent with this hypothesis have been demonstrated in normal human subjects,[4, 5] in patients with congestive heart failure,[4] and those with renal disease without acidosis.[5]

With the exception of a preliminary report[6] and three cases studied by Kaye,[5] no data are available on the effects of carbonic anhydrase inhibitor in the nephrotic syndrome. The present study was carried out (a) to define the results of carbonic anhydrase inhibition in the nephrotic syndrome and (b) to explore further the features of potassium excretion. A carbonic anhydrase inhibitor (2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide,[7] 6063, Diamox*) was infused to block hydrogen-ion production, and subsequently a nonreabsorbable anion load was added to potentiate cation excretion.

*We are indebted to the Lederle Laboratories Division, American Cyanamid Company, New York, for a supply of this material.

EXPERIMENTAL DATA

FILTERED				URINE		EXCRETED						
				VOL. ML./ MIN. /M²								
Na	K	Cl	PO_4		pH	Na	K	NH_4	Cl	HCO_3	PO_4	PAH
	μEQ./MIN./M²					μEQ./MIN./M²						
9,200	220	7,441	197	3.5	6.77	131	35	28	110	19	11	44
7,510	151	5,477	138	4.3	7.56	404	133	4	121	244	28	39
6,540	116	5,269	140	2.9	7.56	506	87	6	73	171	53	390
7,610	248	6,318	144	2.4	6.41	91	38	24	59	5	14	54
4,570	139	4,049	82	4.8	7.59	256	172	5	103	281	16	34
3,870	103	3,456	75	2.5	7.51	294	113	3	52	267	33	210
6,860	206	—	130	2.1	6.41	43	24	—	27	5	20	22
4,670	112	—	94	5.9	7.35	144	77	—	48	140	36	17
4,450	91	—	90	1.8	7.39	252	99	—	8	50	45	241
6,920	199	5,430	202	0.6	5.72	5	49	24	5	0.5	27	28
6,300	205	5,170	175	2.0	7.59	39	151	12	26	44	24	27
4,990	123	4,150	141	1.2	7.14	193	131	9	0	6	34	234
2,460	91	2,010	86	0.2	5.02	1	19	6	5	0	16	14
2,750	91	2,430	92	0.5	7.11	3	78	0.4	8	5	23	20
2,540	75	2,120	93	1.0	6.38	18	182	0.1	3	6	16	152
2,440	76	2,053	—	0.5	5.08	1	19	20	—	0.1	21	22
2,990	87	2,407	—	0.8	7.04	2	97	4	—	14	30	32
2,770	71	2,147	—	1.5	6.60	32	205	8	—	5	25	189
3,390	118	2,730	—	0.1	5.61	1	21	17	11	0.6	15	15
3,470	84	2,820	—	0.7	7.41	8	123	12	32	40	30	23
6,009	151	4,878	—	1.2	5.98	169	203	16	148	12	15	242

METHODS

The control subjects were hospitalized patients awaiting discharge. None had evidence of renal disease, acidosis, or edema.* Like the nephrotic children, they had been either in bed or sedentary for several days and had consumed the ordinary hospital diet (1 to 2 NaCl daily).

The four nephrotic children were very edematous. One of them (L. W.) was studied during a second carbonic anhydrase inhibitor infusion three weeks later when she was nonedematous following steroid-induced diuresis.

The usual clearance procedure[8] was followed, except that blood and urine samples were collected under oil, and the bladder was rinsed with sterile distilled water, but not with air. Following suitable preclearance collection periods, 3 or 4 inulin and PAH clearance periods were obtained. A priming dose (250 mg./M², 9 to 10 mg. per kilogram) of the sodium salt of Diamox was then injected and 450 mg./M² (16 to 20 mg. per kilogram) Diamox was added to the inulin and Na PAH clearance infusate. After 4 to 5 collection periods were completed, a priming dose of 7 to 9 mM/M² (0.25 to 0.35 mM per kilogram) Na PAH was injected, and a solution containing the same concentration of inulin and Diamox as previously, plus 55 to 65 mM/M² (2.0 to 2.5 mM per kilogram) Na PAH infused. Blood was obtained at the start of the procedure and at 20- to 30-minute intervals thereafter. Samples were taken anaerobically at the end of each phase of the study. Each complete study lasted from 4 to 6 hours. Methods were as previously described[1] with the following additions: serum water was determined by desiccation; carbon dioxide content of urine and serum was measured manometrically[9]; a pK′ of 6.1 was used for HCO_3^-; urine pH was measured with a glass electrode in a Beckman pH meter at tempera-

*Patient R. A., diastematomyelia; Patient R. L., extra toe; Patient D. C., history of epistaxis, etiology undetermined.

ture = 24° to 28° C.; and observed values were not corrected to 37° C. since a precise correction factor was not available. NH_4^+ was determined by microdiffusion.[10] Phosphates were determined by slight modification of the method of Fiske and SubbaRow.[11] Serum electrolyte values were corrected for Gibbs-Donnan and binding factors. The "r" values used were as follows: Na and K, 0.94; Cl, HCO_3, and PO_4, 1.05. All excretion and clearance data have been adjusted to 1 square meter surface area. In the edematous children, surface area was estimated from a Dubois nomogram using height and estimated or observed non-edematous weight.

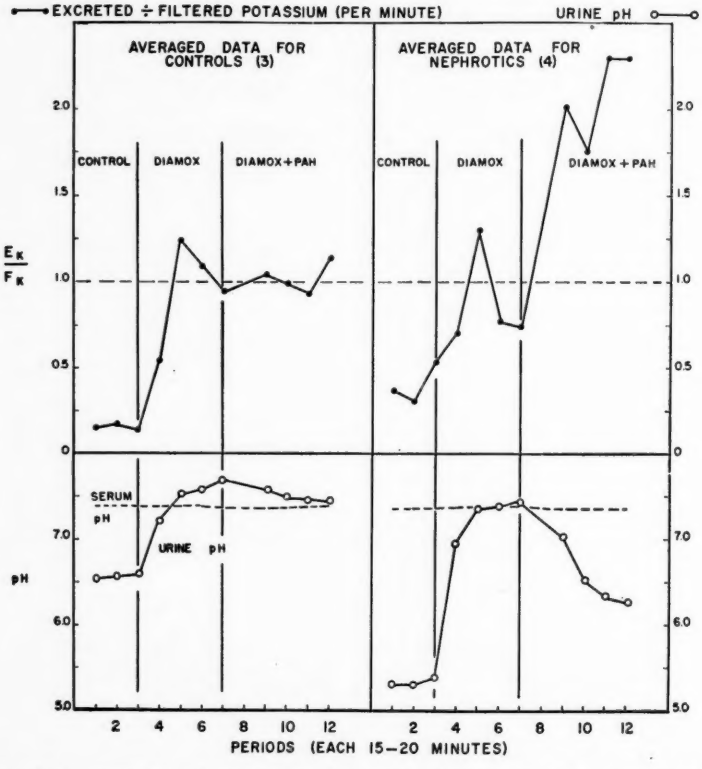

Fig. 1.—Net secretion of potassium by renal tubular cells is demonstrable when the ratio $\frac{EK}{FK}$ exceeds 1.0. The averaged results of each period in the several phases of the study are shown. In the control subjects, potassium secretion is apparent after Diamox and Diamox + PAH. Urine pH remained above that of the plasma. In the nephrotic patients, potassium secretion appeared augmented by addition of the PAH load, and at this time urine pH fell below that of the plasma.

TABLE II. CHANGES IN POTASSIUM AND HYDROGEN-ION EXCRETION

SUBJECTS	INFUSATE	NO. PERIODS	POTASSIUM FILTERED	POTASSIUM EXCRETED	EK* FK	H+†	URINE pH
Control	Control	9	227	32	0.13	3.4	6.53
	Diamox	11	134	124	0.93	9	7.50
	Diamox + PAH	12	103	100	0.97	-3.	7.49
Nephrotic	Control	11	121	27	0.32	25	5.36
	Diamox	15	117	115	1.05	8	7.29
	Diamox + PAH	16	105	180	1.97	10	6.53

*EK = excreted K/min./M²: FK = filtered K/min./M².

†H+ = difference between equivalence of PO_4 at pH of urine and that of the plasma filtrate, plus NH_4^+ excretion.

RESULTS

Averaged data relating to serum and urine electrolytes and renal functions are shown in Table I. Data on potassium and hydrogen excretion are shown in Table II and Fig. 1. The changes in potassium and hydrogen excretion before and after diuresis in Patient L. W. are shown in Fig. 2.

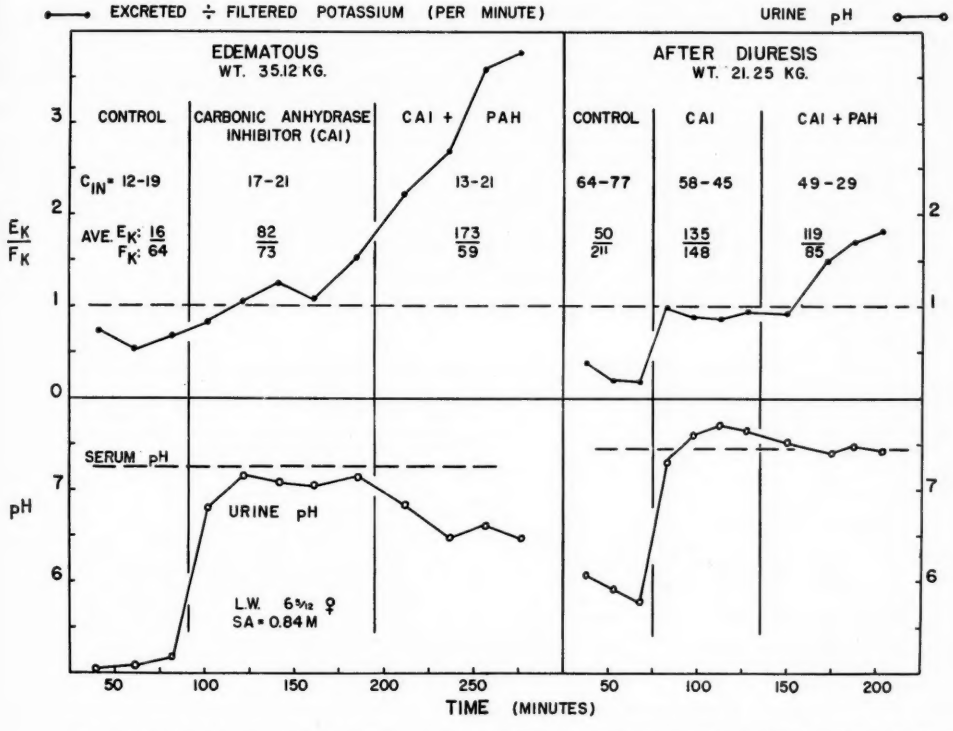

Fig. 2.—Diuresis was induced by administration of ACTH to this patient. Twenty-four days elapsed between the observations on the left and those on the right of the chart. Glomerular filtration rate was normal after diuresis. Note that potassium secretion with Diamox + PAH infusion during edema was associated with increased potassium excretion and an acid urine. After diuresis, potassium secretion accompanied decreased filtration of potassium, and the urine remained alkaline.

Serum Changes.—In all patients but one (E. M.), serum pH remained within the normal range throughout the study. No significant change in pH was noted during infusion of Diamox or Diamox + PAH. In the control subjects there was a uniform fall of 3 mM per liter in serum CO_2 content during infusion of Diamox + PAH. In the nephrotic patients, in whom the CO_2 content was lower initially, this was not observed. Serum sodium concentration remained within the normal range in both groups, but potassium concentration decreased by 0.5 to 1.0 mM per liter during infusion of Diamox and Diamox + PAH in all patients.

Urine Flow and Renal Function.—In the control subjects, urine flow increased following the infusion of Diamox, and inulin and PAH clearance decreased. Since the filtration fraction did not change, decreased clearances

suggested that a fall in renal blood flow had occurred. Infusion of Diamox + PAH resulted in slight, probably insignificant decreases in inulin clearance, and urine flow decreased to control levels or below.

In the nephrotic patients, urine flow was very low during the control periods. With the exception of Patient M. N., inulin clearance was also lower than in the control subjects. Infusion of Diamox resulted in increased urine flow. Little change in inulin clearances were observed, but PAH clearances increased in 3 of 4 patients, indicating a fall in filtration fraction. Diamox + PAH resulted in a further increase in urine flow in 3 out of 4 patients. The effect upon inulin clearance was variable.

Urine Reaction and Electrolyte Excretion.—In the control subjects, Diamox caused a marked increase in the excretion of sodium, potassium, bicarbonate, and phosphate. The pH of the urine became more alkaline than that of the plasma. Infusion of Diamox + PAH caused a further increase in sodium excretion and decreased excretion of potassium in two out of three subjects. The urine pH remained unchanged. Total solute excretion increased, despite a fall in urine flow.

In the nephrotic patients, sodium and chloride excretion were very low during the control period, potassium being the principal urine cation. The urine pH was lower than in the control subjects. Infusion of Diamox resulted in increased sodium and potassium excretion, particularly potassium, and alkalinization of the urine. With Diamox + PAH, sodium excretion was further increased in all patients and potassium excretion was augmented in 3 of 4 studies. The urine pH fell below that of the plasma in all four patients.

Excreted: Filtered, Potassium and Hydrogen Equivalents.—If the quantity of potassium excreted (EK) is divided by the calculated quantity filtered (FK), the quotient indicates the net reabsorption or secretion of potassium by the tubules.* In both control subjects and nephrotic patients, the ratio $\frac{EK}{FK}$ approximated or slightly exceeded one during Diamox infusion. With Diamox + PAH, no further change in this quotient was observed in control subjects, but in nephrotic patients a striking increase occurred, indicating further potassium secretion. The changes in hydrogen-ion excretion in both groups have been calculated from the difference between the equivalents of PO_4 at the pH of urine and of serum plus ammonium excretion. The results indicate increased hydrogen-ion secretion with persistent potassium secretion despite continued administration of Diamox in the Diamox + PAH periods in the nephrotic patients.

DISCUSSION

The changes in urine composition induced by infusion of Diamox in the control children studied were identical to those described in dogs by Berliner[3] and in normal adult human beings by Friedberg and associates[4] and Kaye,[5] using

*For convenience, the term "potassium secretion" is used only when the fraction $\frac{EK}{FK}$ exceeds unity. It is not intended to suggest that tubular secretion of potassium occurs *only* under these conditions. The relative importance of reabsorption vs. secretion in potassium excretion is not yet established.

either oral or intravenous administration of the drug. Decreased serum potassium concentration during Diamox administration suggests that the rate of potassium loss into the urine from the extracellular phase may be greater than that of its replenishment rate from other sources. Decreased inulin clearance during Diamox infusion has been noted by Berliner.[3]

Carbonic anhydrase inhibition, evidenced by the production of an alkaline urine, also occurred in the children with the nephrotic syndrome. In contrast with the control subjects, however, potassium was the principal urine cation. Similar, but less striking, changes have been demonstrated in patients with a variety of renal diseases without acidosis who were given Diamox orally.[5] In patients with congestive heart failure given Diamox, resultant potassium excretion was approximately equal to sodium excretion when the previous dietary sodium intake was low.[4] Sodium excretion was much greater when the previous intake had been normal, but potassium secretion was unchanged. The relatively slight increase in sodium excretion observed in the nephrotic children in response to Diamox may account for the generally poor results obtained from its use as a diuretic in this disease.

The results of PAH loading + Diamox may be compared to those obtained previously[1] when PAH loading alone was performed. In normal children, excretion of a nonreabsorbable anion load was effected with an equivalent quantity of fixed cation, principally sodium. Urine pH remained acid. When, in the present study, loading was superimposed upon carbonic anhydrase inhibition, a further increase in sodium excretion occurred. The urine remained alkaline, and potassium excretion decreased in two out of three patients.

The results of nonreabsorbable anion loading were quite different in the edematous nephrotic children. Without Diamox, excretion of the load was covered by approximately equal quantities of potassium and sodium; urine pH remained acid.[1] Net potassium secretion was usually observed. In the present study, loading caused a further increase in both sodium and potassium excretion, but the urine pH decreased significantly. In one patient (L. W.) who was studied following diuresis when she was nonedematous, urine pH became alkaline with Diamox and did not decrease with subsequent addition of PAH to the infusate. However, net potassium secretion persisted, this result being similar to those in the previous study, where the secretion persisted following diuresis, although sodium excretion reverted to normal.

The acidification of the urine observed in the nephrotic children during Diamox + PAH loading might be due to (a) a decreased quantity of Diamox reaching the tubule cell, (b) decreased effectiveness of Diamox, (c) H^+ secretion by some mechanism not affected by Diamox. Although plasma Diamox assays were not performed, similar amounts proportional to weight were infused at the same rates in nephrotic and control children. The greater reduction in renal blood flow observed in the latter tended to equalize the amount of Diamox reaching the tubule cells per unit time in both groups. The amount used caused satisfactory inhibition of hydrogen-ion secretion during the first phase of the study, and Diamox is not very rapidly destroyed or

excreted.[12] The relationship between quantity of Diamox infused, renal blood flow and tubular carbonic anhydrase inhibition requires further definition.

The degree of metabolic acidosis observed was probably insufficient to cause inhibition of Diamox effect[5] or to increase significantly the amount of hydrogen ion reaching the urine by filtration of carbonic acid. It appears likely therefore that the effectiveness of carbonic anhydrase inhibition was reduced or some other means of providing hydrogen secretion was activated in the face of acute demand for cation to cover the anion load. Transport of hydrogen across cell membranes to satisfy cation equivalents has been postulated during alkalosis[13-15] and acidosis.[15, 16] The present findings also suggest that hydrogen ions were provided by renal tubular cells in support of cation requirements. Since acidification of the urine was observed while carbonic anhydrase inhibition presumably was being maintained and potassium secretion augmented in the nephrotic patients, it seems likely that the net hydrogen-ion content of the urine was increased by some mechanism other than that occurring in normal subjects on an unrestricted sodium intake which involves competition between hydrogen and potassium for a sodium transport pathway.[3] It is possible that normal subjects stimulated to maximal renal conservation of sodium by restriction of sodium intake might respond to carbonic anhydrase inhibitor like the nephrotic children. Whether tubular transport of PAH, per se, in the nephrotic patient augments hydrogen-ion secretion, or some other relationship dependent upon the homeostatic removal of potassium and retention of sodium[1] is responsible for this phenomenon, cannot be resolved until the nature of these ion-transport mechanisms within the cell and their relationship to one another is clarified.

SUMMARY

A carbonic anhydrase inhibitor (Diamox) was infused in four nephrotic children and three control subjects. Infusion resulted in an alkaline urine and increased cation excretion. Potassium was the principal cation excreted in the nephrotic patients, sodium in the controls.

When a nonreabsorbable anion (PAH) load was added to the infusate, acidity of the urine increased in the nephrotic patients and net potassium secretion by the tubules was demonstrated. These changes did not occur in the control subjects.

The results suggest that other mechanisms for acidification of the urine are present during periods of increased sodium reabsorption in nephrotic edema in contrast to the demonstrable competition between hydrogen and potassium for some common pathway of sodium exchange present in control subjects. This unusual form of hydrogen excretion in nephrotic patients provides further evidence for altered renal tubular electrolyte transport, presumably in defense of body fluids, in this form of edema.

We wish to acknowledge the technical assistance of Antoinette DeSimone and Mary Gadway, R.N.

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DIASTOLIC OVERLOAD IN MECHANICAL VENTRICULAR ALTERNANS

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WITH THE TECHNICAL ASSISTANCE OF GLADYS HECKMAN, R.N., AND HANNA JANOUSKOVEC, R.N.

PRESENT experimental evidence has not yet clearly separated the relative importance of myogenic and hemodynamic factors in the genesis of mechanical ventricular alternans.[9] Two recent reports dealing with pulsus alternans have supported the view that ventricular alternation is more likely to be secondary to changes in venous return and peripheral resistance,[1, 4] with resultant hemodynamic changes, than to primary aberrations in myocardial contractility.

Chance observations recorded during a mild exercise experiment on a patient with decompensated aortic stenosis appear to emphasize the importance of the dynamic factors, though failing to adduce conclusive evidence dissociating the role of primary myogenic changes. The data likewise indicate that changes in total end-diastolic overload may be independent of alterations in the venous return in the production of mechanical alternation of the ventricles.

MATERIAL AND METHODS

H. B., a 64-year-old Negro man, was seen early in February, 1955, at Crile Veterans' Administration Hospital, because of congestive heart failure.* For three years prior to admission he had experienced exertional dyspnea, paroxysmal nocturnal dyspnea, and several episodes of syncope. The physical findings included a diffuse increase in palpable left ventricular activity, with the apical beat visible just within the anterior axillary line in the sixth intercostal space, and a loud, harsh, slightly musical, "diamond shaped," holosystolic murmur heard best over the second right parasternal area, transmitted to the apex and to both carotid arteries. There was present a systolic thrill bilaterally over the upper parasternal areas, and the second aortic sound could not be heard in the second right parasternal space. No diastolic murmurs were present. The liver edge was palpable several finger-breadths below the costal margin. Edema of the lower extremities extended to the hips. In the teleoroentgenogram of the chest there was more than 25 per cent enlargement of the transverse diameter of the cardiac silhouette. The electrocardiograph inscribed left axis deviation, a horizontal position, counterclockwise rotation, and depressed ST segments, inverted T waves, tall R waves, and delayed onset of the intrinsicoid deflection in the left precordial leads. There was no evidence of old or recent myocardial infarctions.

The patient responded well to routine digitalization and treatment, and had been reduced to "dry weight" for one week at the time of investigation. On Feb. 16, 1955, cardiac catheterization and jugular bulb puncture were performed, in order to assess the effects

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upon general and cerebral blood flows of mild, steady state exercise. The latter consisted of bicycle pedal exercise against a variable resistance ergometer,* in the supine position, employing a low work rate of 110 footpounds (40 revolutions) per minute. The methods employed, except for the estimation of the cerebral blood flow and the details of work performance, were as previously reported.[6]

Following the basal determinations during which no alternation was present (Fig. 1), exercise was begun. At the 5-minute period a pulsus alternans was noted. This continued virtually unchanged throughout the balance of a 32-minute period of exercise, and disappeared 5 minutes after the cessation of exercise. Two other sets of pressure pulses, recorded during the 14- and 28-minute periods, are presented in Figs. 2 and 3. The essential data are recorded in Table I. The respiratory rate was 20 and the electrocardiograph recorded a sinus mechanism throughout, ruling out the confusing possibility of a pseudoalternans. Pulsus alternans had not been previously noted in this patient, nor was it produced by exercise of a similar order in 24 other patients with aortic lesions similarly investigated.[5]

TABLE I. CATHETERIZATION DATA RELEVANT TO PATIENT H. B.

	CONTROL	10-17 MIN. EX.	24-32 MIN. EX.
H. R.	94	100	100
R. R.	16	20	20
R. A.	12.4/10.5	--	15.3/12.2*
R. A. M. P.	11.2	--	13.1
R. V.	56/11	--	69/15*
R. V. M. P.	25.1	--	32.7
P. A.	55/22	70/32	67/30
P. A. M. P.	35.5	46.4	42.5
W. P. ('P. C. P.')	14.1	18.1	17.7
B. A.	136/88	142/91	144/93
B. A. M. P.	106.0	107.3	111.4
$O_2C.$—1	194	280	336
$O_2C.$—2	198	288	330
$O_2C.$—Average	196	284	333
C. O.—1	3119	3087	3876
C. O.—2	3177	3180	3820
C. O.—Average	3148	3133	3848
Stroke volume	33.5	31.3	38.5
A-V D.—1	6.22	9.07	8.67
A-V D.—2	6.24	9.05	8.64
A-V D.—Average	6.23	9.06	8.65
Pu. T. R.	902	1184	867
E. T. P. R.	2477	2738	2316

Pressures in millimeters of mercury; mean pressures derived by planimetric integration. All pressures represent averages of 3 consecutive respiratory cycles. Oxygen consumption (O_2C) in milliliters per minute, cardiac output (C.O.) in milliliters per minute, A–V difference (A-V D.) in volumes per cent of oxygen, resistances (Pu.T.R., total pulmonary, E.T.P.R., peripheral), in c.g.s. units.

*Pressures recorded during the final minute of exercise.

RESULTS

That the patient was still in failure at the time of the experiment, though this was clinically unapparent, may be noted by the elevated right atrial, the right ventricular end-diastolic, and pulmonary wedge pressures, recorded among the control data in Table I. At this time only the normal respiratory variation

*Manufactured by Gilmore Industries, Inc., 5713 Euclid Ave., Cleveland, Ohio.

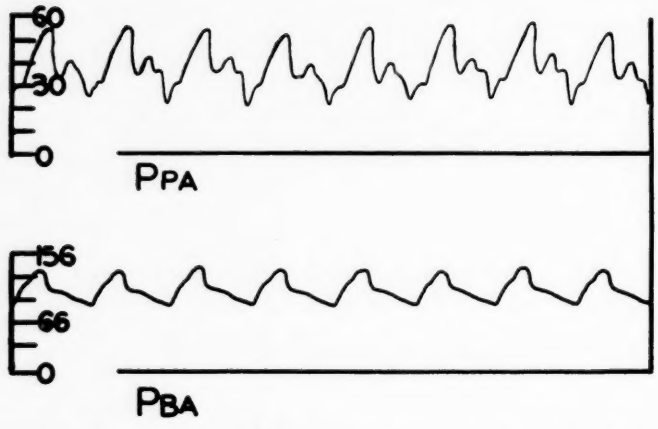

Fig. 1.—Control data. P_{PA} is pulmonary artery pressure, P_{BA} is brachial artery pressure in millimeters of mercury. Scale: P_{PA}, 1 mm. deflection is 2 mm. Hg; P_{BA}, 1 mm. deflection is 6 mm. Hg. The electrocardiogram, demonstrating sinus mechanism without premature beats or electrical alternans, is not included for convenience. In both tracings only the normal respiratory variation is noted.

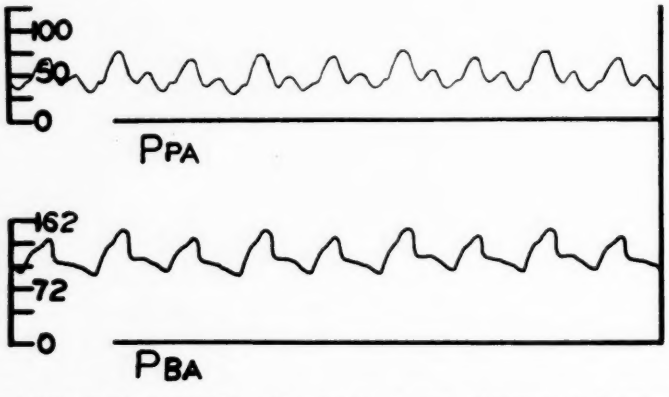

Fig. 2.—Intermediate stage of exercise. P_{PA} scale: 1 mm. deflection is 5 mm. Hg; P_{BA} scale as above. Note alternation from both ventricles.

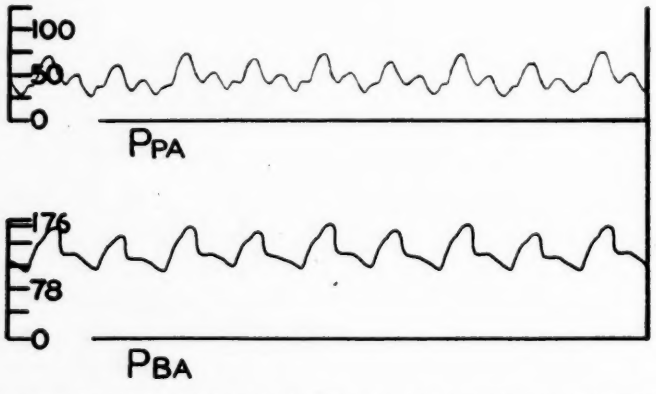

Fig. 3.—Final stage of exercise. Scale as in Fig. 2. Note continued alternation. (See text.)

is seen in the pulmonary and brachial arterial pressure pulses (Fig. 1). In Fig. 2, recorded midway between two check cardiac outputs determined from the 10- to the 17-minute periods of exercise, an average systolic alternans of 7.2 mm. Hg in the pulmonary and 10.1 mm. Hg. in the brachial arterial pressure pulses is seen. Pulmonary artery end-diastolic pressure has risen from 22 to 32 mm. Hg, pulmonary artery wedge pressure from 14.1 to 18.1 mm. Hg, and brachial arterial pressure is slightly elevated. This occurs in the face of an unchanged venous return, evidence of the lack of cardiac reserve. Regardless of the inability to achieve steady state throughout the 10- to 32-minute periods of exercise, the closeness of each set of check determinations for arteriovenous oxygen differences and oxygen consumptions validates the accuracy of the calculated cardiac outputs during the control, intermediate, and final stages of exercise.

During the 24- to 32-minute periods, pulmonary artery end-diastolic and wedge pressures are virtually unchanged from the intermediate levels. Right ventricular end-diastolic pressure has increased from 11 (control) to 15 mm. Hg, and right atrial pressure from 12.4/10.5 (control) to 15.3/12.2 mm. Hg. At this time, in association with the increased ventricular filling pressures, the cardiac output has increased noticeably, and compensation for the increased oxygen consumption is no longer solely dependent upon increased venous oxygen extraction (decreasing A-V oxygen difference). The degree of alternation, both right and left ventricular, remains virtually unchanged, being in the order of 8.5 and 9.0 mm. Hg, respectively (Fig. 3). No attempt was made to influence the alternans by increasing the work rate, and the experiment was terminated.

DISCUSSION

It is worthy of note that in this patient alternation appeared with no change and persisted in spite of a measurable increase in the venous return. The observations of Friedman and associates[1] emphasize the importance of orthostatic factors which *diminish* the venous return in the production or accentuation of the alternans, whereas measures which would be expected to increase this return tend to abolish or reduce it. Ryan and associates,[4] on the other hand, have shown that pulsus alternans may disappear as congestive failure progresses in severity, a situation in which there is good reason to believe that the venous return progressively *decreases.*[7] In their cases, however, no measurements of the cardiac output were made. The concept of both groups is that with an increase in ventricular end-diastolic pressure and volume, consequent to changes in the venous return, a more equal stretch of the myocardial fibers from cycle to cycle is produced. This is stated to lead to a diminution or disappearance of the alternans, by increasing the degree of ventricular stretch and eliminating the unequal residual volumes[1] at the end of the weak and strong beats. These authors,[1] although unable directly to measure cycle-to-cycle variations in ventricular end-diastolic volume, presented

supportive evidence for such changes based on electrokymographic observations. These are confirmatory of the more direct observations of Straub[8] and the volume curve analyses of Wiggers.[9]

The data herein included, though not measuring intraventricular volumes, are in no way at variance with the previous reports in so far as hypothecated dynamic intraventricular changes are concerned. Rather, they do emphasize that *directional* changes in venous return are not as important a factor in the appearance or disappearance of pulsus alternans, as are the changes in ventricular end-diastolic pressure and, inferentially, end-diastolic volume and initial stretch. The amount of the venous return may be variable and bidirectional. It is seen that alternation may occur in situations where the venous return presumably decreases,[1] remains unaltered, or increases (present observations).* Admittedly, none of the evidence available to date clearly separates changes in diastolic overload and possible primary alterations in myocardial contractility, in as much as data relating to changes in myocardial metabolism are not at hand. It is none the less difficult to lend too much importance to primary alterations in myocardial metabolism and contractility, when one considers that any possible deleterious myogenic influences leading to the appearance of alternation, as occurred in Ryan's patients, would likely have become worse with the unabated progression of congestive failure which was accompanied by eventual disappearance of the alternans.

From the observations of both groups,[1, 4] and the data herein reported, it may be hypothesized that pulsus alternans occurs *over a critical range of increased ventricular end-diastolic volumes,* making an appearance at a level above the normal value, and eventually disappearing at a further increased level. It is likewise probable that variability in the appearance of alternation occurs in different subjects, and while we feel that our data confirm the importance of the dynamic factors involved, they do not deny the possible coexistence of primary myogenic factors in this type of alternation, and their importance in that form which occurs during experimental coronary artery occlusion.[2, 3]

SUMMARY AND CONCLUSIONS

1. Mechanical pulsus alternans has been produced in a patient with decompensated aortic stenosis during the performance of mild exercise. This occurred in association with further increases in previously abnormally elevated ventricular end-diastolic pressures and persisted during periods when the venous return was unchanged from the control pre-exercise values, and later when increased above them.

2. Previous reports have emphasized the influence of changes in the venous return as determinants of alterations in diastolic overload of the ventricles. The present data point more to the importance of primary end-diastolic

*Ryan has recently observed two patients in whom exercise produced mechanical ventricular alternation. Unfortunately, data for the calculated cardiac outputs are not available. (Ryan, J. M.: Personal communication.)

intraventricular pressures and, by inference, end-diastolic volumes, which may be independent of primary alterations of the venous return. The latter may be unchanged or bidirectional.

3. It is further suggested that alternans appears at variable levels of increased end-diastolic pressure, and may later disappear as the latter increases further over a critical range. As inferred from the observations of others, this may be brought about either by the induction of a sudden increase in the venous return, or in the face of progressive decreases in venous return associated with progressive, intractable congestive failure.

4. The data presented, as well as the reports of others, do not dissociate primary myogenic changes from hemodynamic factors in the genesis of ventricular alternans. However, they do support the thesis that changes in diastolic overload are of importance and, conceivably, independent from such myogenic factors.

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THE EVALUATION OF BRONCHODILATOR DRUGS IN THE TREATMENT OF ASTHMA

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THE evaluation of drugs in the treatment of bronchial asthma presents considerable difficulty. Many of the difficulties stem from the greatly varying pathology of the condition. We define asthma as a syndrome, consisting of paroxysmal and recurrent partial obstruction of the lower bronchial tree, resulting in the development of acute diffuse obstructive emphysema. The obstruction of the lower respiratory tract may result from spasm of the bronchial musculature, congestion and edema of the bronchial mucosa, or narrowing of the bronchial lumen because of the presence of viscid secretion. When the process has been long standing, intramural inflammatory changes occur. The superimposition of infection will change the character and quantity of secretion and may result in additional changes in the degree of mucosal edema, congestion, and cellular infiltration. The relative importance of these various factors will vary greatly from patient to patient and in the same patient at different times.

Simple clinical observations and the subjective impressions of patients have been widely used in evaluating the efficacy of drugs in relieving asthma. If a drug has a dramatic effect during an acute asthmatic paroxysm, there can be little question as to its benefit. Less dramatic effects are much more difficult to evaluate. Physical examination bears little quantitative relationship to the severity of asthma and correlates poorly with objective tests of ventilatory function. The subjective impressions of patients are also notably unreliable. This was quite apparent in our study of the effects of intramuscular visammin as a bronchodilator.[1] Six patients who were in the midst of acute asthmatic paroxysms were given an intramuscular injection of the drug which was bright red in color. The injection was quite painful and was accompanied by positive suggestion. In this admittedly "loaded" situation, all patients reported partial relief of their dyspnea, but objective tests of ventilatory function failed to confirm this subjective impression. Another point is that, in our experience, it is usually difficult to detect mild or moderate degrees of bronchial obstruction except by some objective laboratory method.

Many laboratory techniques have been employed to delineate the physiologic changes which occur in asthma.[2] Measurements of ventilatory function

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such as the vital capacity and its modifications, and the maximum breathing capacity test, have been most widely used in evaluating the effects of drugs in the laboratory.

The vital capacity measures the maximal volume of air which can be moved in a single expiratory effort irrespective of time. It is a poor measure of airway obstruction since it remains normal until the condition is severe. The maximum breathing capacity is a voluntary test of maximum ventilation expressing the largest volume of air which can be moved in a unit of time. This is considered by most observers to be the best measure of true maximum ventilatory ability and permits a good estimate of ventilation reserve. The maximum breathing capacity relates volume and time of air flow and is therefore a much better measure of bronchial obstruction than the vital capacity. These tests have been well standardized in normal persons and formulas are available for prediction of expected values in any patient on the basis of height, weight and age.[3]

A number of observers have attempted to overcome the deficiencies of the vital capacity by making observations of both volume and velocity of air flow during the performance of the test. The volume of the vital capacity may remain normal, and airway obstruction may be demonstrated only by changes in the expiratory slope of the curve. Bruce[4] divided the volume of the vital capacity by the recorded elapsed time and obtained "vital rates" which varied from 3 to 8 liters per second in normal subjects. Gaensler[5] analyzed the vital capacity using a simple vital capacity spirometer which had been modified by the addition of an electronic timer. The timing cycle was initiated by the onset of expiration. This instrument could be pre-set to measure the volume of air moved during the first 0.1 to 10 seconds of a forced expiration. The volumes showing the largest variations between normal volunteers and patients were those measured during the first, second, and third seconds. These were designated the "1, 2, and 3 second vital capacities." Normal persons, on the average, expired 83 per cent of the total vital capacity in the first second of the effort; 94 per cent, during the first two seconds, and 97 per cent, during the first three seconds. There was a high correlation between the one second vital capacity expressed as per cent of predicted vital capacity and the maximum breathing capacity expressed as per cent predicted. The correlation between the maximum breathing capacity and the 2 and 3 second vital capacities was lower.

These tests of ventilatory function have been used in a number of ways to evaluate antiobstructive drugs. The groups of Curry[6, 7] Lowell[8, 9] and Segal[10-13] have studied the ability of drugs to protect against the development of asthmalike attacks in susceptible subjects following the administration of histamine, acetyl-beta-methylcholine, or aerosolized extracts of allergens. They gauged the severity of attacks and the degree of protection afforded by a drug by means of vital capacity tests repeated at brief intervals. Gaensler[14] has pointed out that the vital capacity may give a false indication of the

actual protection afforded the patient. In one of his studies the vital capacity indicated almost 100 per cent protection by isopropyl Arterenol aerosol against histamine-induced asthma. Maximum breathing capacity tests performed simultaneously showed only 17 per cent protection.

Protection studies have afforded valuable information on the comparative effects of drugs under laboratory conditions. They have the advantage of providing a technique which may be repeated with fairly good reproducibility at the convenience of the investigator. However, the results of these studies must be applied to clinical asthma with great caution. The main justification for so doing is that asthmalike attacks can be induced by the above methods in asthmatic or hayfever patients but not in normals.[6] On the other hand, these induced attacks differ from one another and from clinical asthma. Antihistaminic drugs provide effective protection against histamine-induced asthma but not against acetyl-beta-methylcholine-induced asthma.[13] The reverse is true of atropine.[11] Neither agent is effective against allergen-induced asthma,[8] and these agents have been of little help in clinical asthma.

The maximum breathing capacity has been criticized as a test of ventilatory function in asthmatics on the following grounds: An occasional patient is encountered where maximum breathing capacity determinations cannot be performed because they result in marked aggravation of the asthma. The test is a strenuous one and cannot be performed by the most seriously ill patients. It has been stated that it may be impossible to obtain reliable results in acute asthmatics because of the occurrence of fatigue as a result of the tests and the increase in airway obstruction resulting from forced expiration during the test. It has been suggested that the timed vital capacity may well fill the need for a less strenuous procedure to replace the maximum breathing capacity. For several years we have been carrying out studies on the efficacy of drugs in asthma. These studies have all been performed in patients with spontaneous asthmatic attacks because of the disadvantages of protection studies discussed previously. We have reviewed some of our data in an attempt to evaluate the adequacy of our technique and to investigate the relative merits of the maximum breathing capacity and the 1 second vital capacity.

METHOD

Laboratory Techniques.—We have used the vital capacity, the 1 and 2 second vital capacities, and the maximum breathing capacity tests in studying asthmatics. The 1 and 2 second vital capacities and the vital capacities were measured using a simple water spirometer fitted with a Gaensler-Collins electronic timer. The vital capacity and 1 second vital capacity were expressed in liters of air at room temperature, wet, ambient pressure. These determinations always preceded measurement of the maximum breathing capacity. The maximum breathing capacity was determined using a 9 liter Benedict-Roth spirometer* with soda lime tower and valves removed. A 15 second interval was used and results were expressed in liters per minute, at 37° C. wet, ambient pressure.[3] Measurements of maximum breathing capacity, vital capacity, and timed vital capacity were repeated until good agreement between duplicate determinations for each test was obtained. The largest measurement was recorded. Predicted values were calculated according to age, sex, height, and weight.[3] Patients were selected for study from the allergy clinic

*Warren E. Collins, Inc., Boston, Mass.

and from the emergency room of the hospital. A few patients were encountered whose asthma was markedly aggravated by the maximum breathing capacity and a small number of patients were unable to perform this test with sufficient reliability. These were discarded from the study. These objective techniques have been used in a long-term study and in an acute study in our attempts to evaluate bronchodilator drugs.

Long-Term Study.—Patients with chronic asthma were selected. They were all known to respond to subcutaneous epinephrine or Isuprel aerosol (1:200) using the maximum breathing capacity and 1 and 2 second vital capacity to evaluate airway obstruction. The drug to be tested was given orally several times daily for two-week periods, alternating with two-week periods on placebo. A "double blind" technique was employed in which neither investigator nor patient knew the identity of the medication. The patients were instructed to use only epinephrine subcutaneously or epinephrine 1:100 aerosol for the relief of acute asthmatic paroxysms. A diary was kept of the number of attacks, the amount of cough and expectoration, and the number of treatments with epinephrine. The ventilatory function tests and clinical evaluation were repeated weekly during the study period of from four to twelve weeks.

Acute Study.—Patients were brought to the laboratory during asthmatic paroxysms of varying severity and the following serial determinations were made. Control determinations of the maximum breathing capacity, the vital capacity, and the 1 and 2 second vital capacities were made during the attack. The patients were given the drug to be tested and, after a suitable interval which depended on the drug used, the determinations were repeated. A known bronchodilator drug, usually 0.3 mg. of epinephrine subcutaneously, was then administered and 20 to 30 minutes later a new set of determinations was repeated. Patients were not included in the study if reversible airway obstruction could not be demonstrated either after the drug to be tested, or after the known bronchodilator.

RESULTS

Long-Term Study.—The long-term study has, to date, yielded results which are difficult to evaluate. Objective tests, as we used them, appear to have added little to clinical evaluation. The weekly tests of ventilatory function have frequently been at variance with the results reported for the previous week by the patient. Repeated studies during periods when the patients were on drug or placebo have not been consistent. These findings are interpreted as an indication of the great day-to-day variability of asthma. We should not expect a single observation of ventilatory function to mirror accurately the course of a whole week. More frequent testing of ventilation during the course of the study may make objective methods more useful in such long-term studies.

Acute Study.—The method of acute study has yielded much more clear-cut results than the long-term study. We use this technique first when evaluating a new drug for asthmatic patients.

It has already been pointed out that we have encountered a few patients who were too ill to perform the maximum breathing capacity test reliably, or in whom the asthma was made worse by the test. We have tried to evaluate the relative variability and accuracy of the maximum breathing capacity, and 1 second vital capacity, and the factor of fatigue in the performance of these tests. Data obtained during study of oral choline-theophyllinate* was reviewed for this purpose. Three sets of serial duplicate studies were performed

*Choledyl—Nepera Chemical Company, Nepera Park, Yonkers, N. Y.

in each of 21 patients as follows: (1) a control study before any drug was given; (2) a study one hour after the oral administration of the drug (Choledyl); and (3) a study 20 to 30 minutes after the administration of 0.3 mg. epinephrine subcutaneously. The entire study covered a period of approximately two hours.

The degree of asthma was severe in most of these patients as indicated by the control maximum breathing capacity which was less than 50 liters per minute (17 to 44 liters per minute) in 15 of the 21 cases. The mean variability of duplicate determinations of the maximum breathing capacity for the entire group was low; it was 2.3 ± 2.3 liters per minute, or less than 5 per cent of the determined maximum breathing capacity. It was noted, however, that the variability became progressively larger as the determined maximum breathing capacity increased in magnitude. This is shown in Table I where the 63 studies have been divided into two groups. Group I consisted of 36 studies where the maximum breathing capacity was less than 50 liters per minute, and Group II consisted of 27 studies where the maximum breathing capacity was greater than 50 liters per minute. Although small numerically, the mean difference between duplicate maximum breathing capacities in Group II is almost three times as large as in Group I, and this difference is statistically significant ($p. < 01$). The mean variability of the 1 second vital capacity was 48 ± 60 c.c. or 5.2 per cent of determined 1 second vital capacity in the entire series. As shown in Table I the variability rose slightly as the degree of airway obstruction decreased, but this difference was not statistically significant. In order to obtain more comparable units the mean difference between duplicate maximum breathing capacities and 1 second vital capacities are also expressed as a percentage of determined maximum breathing capacity and 1 second vital capacity. The order of magnitude of variability is the same for both measurements.

The effect of fatigue in the performance of repeated maximum breathing capacities is difficult to evaluate. Table II shows the mean difference between duplicate determinations of maximum breathing capacity and 1 second vital capacity determinations in the 21 cases of the choline-theophyllinate study. The mean variability of the maximum breathing capacity was higher after the test drug than in the control study, and still higher after epinephrine. Part of the increase in variability is due to the fact that the maximum breathing capacity was higher after the drug and after epinephrine than at the control period. But even when the data is classified according to the size of the maximum breathing capacity at each of the three study points (Table III) this difference in mean variability persists. These latter differences were numerically small and not statistically significant, but fatigue cannot be excluded as a factor in their causation. The 1 second vital capacities also show slight increase in variability as one progresses through the study.

In order to study the relative reliability of the maximum breathing capacity and 1 second vital capacity, 108 "simultaneous" observations of maximum breathing capacity and 1 second vital capacity made during 36

studies on choline-theophyllinate were compared. We found a high correlation between the maximum breathing capacity and the 1 second vital capacity. The coefficient was exactly the same (0.83 ± .02) whether the correlations were performed with the raw data expressed in liters per minute and liters for the maximum breathing capacity and vital capacity, respectively, or whether the maximum breathing capacity was expressed as a percentage of predicted maximum breathing capacity, and the 1 second vital capacity as a percentage of predicted vital capacity.

TABLE I. MEAN DIFFERENCE, WITH STANDARD DEVIATIONS, BETWEEN 63 DUPLICATE DETERMINATIONS OF MAXIMUM BREATHING CAPACITY AND 1 SECOND VITAL CAPACITY

	MAXIMUM BREATHING CAPACITY		1 SECOND VITAL CAPACITY	
	LITERS PER MINUTE	% DETERMINED M.B.C.	LITERS	% DETERMINED 1 SEC. V.C.
Group I Maximum Breathing Capacity <50 L./Min. 36 studies	1.3 ± 1.82	4.3	.04 ± .055	5.9
Group II Maximum Breathing Capacity >50 L./Min. 27 studies	3.7 ± 2.35	5.3	.06 ± .049	4.2
Total 63 studies	2.3 ± 2.3	4.7	.048 ± .060	5.2

TABLE II. MEAN DIFFERENCE WITH STANDARD DEVIATIONS BETWEEN DUPLICATE DETERMINATIONS OF MAXIMUM BREATHING CAPACITY AND 1 SECOND VITAL CAPACITY AT THREE OBSERVATION POINTS IN 21 PATIENTS*

CONTROL		AFTER CHOLEDYL		AFTER EPINEPHRINE	
MAXIMUM BREATHING CAPACITY	VITAL CAPACITY 1 SECOND	MAXIMUM BREATHING CAPACITY	VITAL CAPACITY 1 SECOND	MAXIMUM BREATHING CAPACITY	VITAL CAPACITY 1 SECOND
1 1.3 L./Min. 2.5% M.B.C.	40 ± 50 c.c. 4.4% V.C.	1.4 ± 3.8 L./Min. 3.2% M.B.C.	48 ± 60 c.c. 4.8% V.C.	3.2 ± 2.2 L./Min. 5.3% M.B.C.	62 ± 67 c.c. 4.7% V.C.

*The differences are expressed both in liters per minute or liters and as a per cent of determined maximum breathing capacity and 1 second vital capacity.

TABLE III. MEAN DIFFERENCE BETWEEN DUPLICATE DETERMINATIONS OF MAXIMUM BREATHING CAPACITY AND 1 SECOND VITAL CAPACITY AT THREE OBSERVATION POINTS IN 21 PATIENTS.*

M.B.C.	CONTROL			AFTER CHOLEDYL			AFTER EPINEPHRINE		
	NO. CASES	M.B.C.†	1 SEC. V.C.‡	NO. CASES	M.B.C.	1 SEC. V.C.	NO. CASES	M.B.C.	1 SEC. V.C.
Group I <50 L./Min.	15	0.5 L./Min. 1.6% M.B.C.	40 c.c. 5.7% V.C.'§	13	1.7 L./Min. 6.0% M.B.C.	40 c.c. 5.7% V.C.'	8	2.6 L./Min. 7.2% M.B.C.	50 c.c. 6.9% V.C.'
Group II >50 L./Min.	6	2.3 L./Min. 3.4% M.B.C.	50 c.c. 3.5% V.C.'	8	5.0 L./Min. 7.4% M.B.C.	80 c.c. 5.3% V.C.'	13	3.7 L./Min. 4.9% M.B.C.	80 c.c. 4.7% V.C.'

*The determinations have been divided into Group I, where the Maximum Breathing Capacity is <50 L/Min. and Group II, where it is >50 L/Min.
†Maximum breathing capacity.
‡1 second vital capacity.
§% determined 1 second vital capacity.

The observations of maximum breathing capacity and 1 second vital capacity at the control point after Choledyl and after epinephrine were then plotted for each individual case, so that their comparability in indicating bronchodilation could be assessed. We found that the 36 studies could be divided into three groups. Group I consisted of 24 cases in which the maximum breathing capacity and 1 second vital capacity ran an approximately

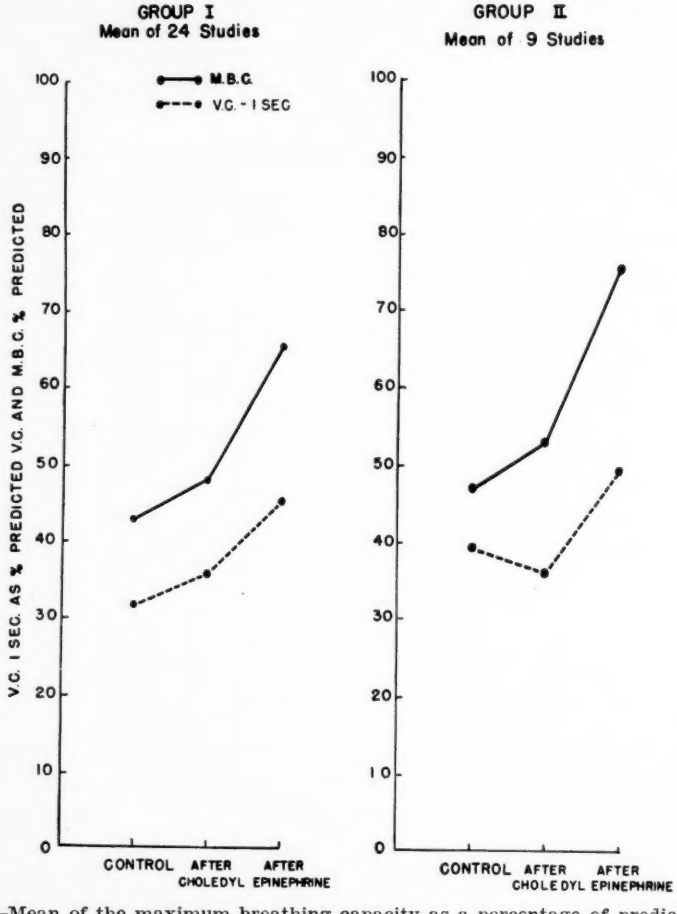

Fig. 1.—Mean of the maximum breathing capacity as a percentage of predicted and 1 second vital capacity as a percentage predicted vital capacity plotted for the control point, after oral choline theophyllinate and after epinephrine. Group I consists of 24 cases in which the maximum breathing capacity and 1 second vital capacity ran a parallel course. Group II consists of 9 cases where the 1 second vital capacity failed to rise, or fell at one observation point.

parallel course. Group II consisted of 9 cases in which the 1 second vital capacity failed to rise or actually fell at one observation point when the maximum breathing capacity rose. This disparity occurred most often after Choledyl. Group III consisted of 3 cases in which the maximum breathing capacity fell or did not rise at one or both observation points when the 1 second vital capacity showed a rise. The means of the maximum breathing capacity and 1 second vital capacity for the 3 observation points are plotted for Groups I and II in Fig. 1 and the individual cases of Group III are shown

in Fig. 2. The question arises as to whether in Group II the 1 second vital capacity or the maximum breathing capacity reflects the true state of affairs. While the maximum breathing capacity may not be a true maximum and can therefore be falsely low, it cannot be falsely high. It therefore follows that in these 9 cases the maximum breathing capacity demonstrated an antiobstructive effect which was not demonstrated by the 1 second vital capacity.

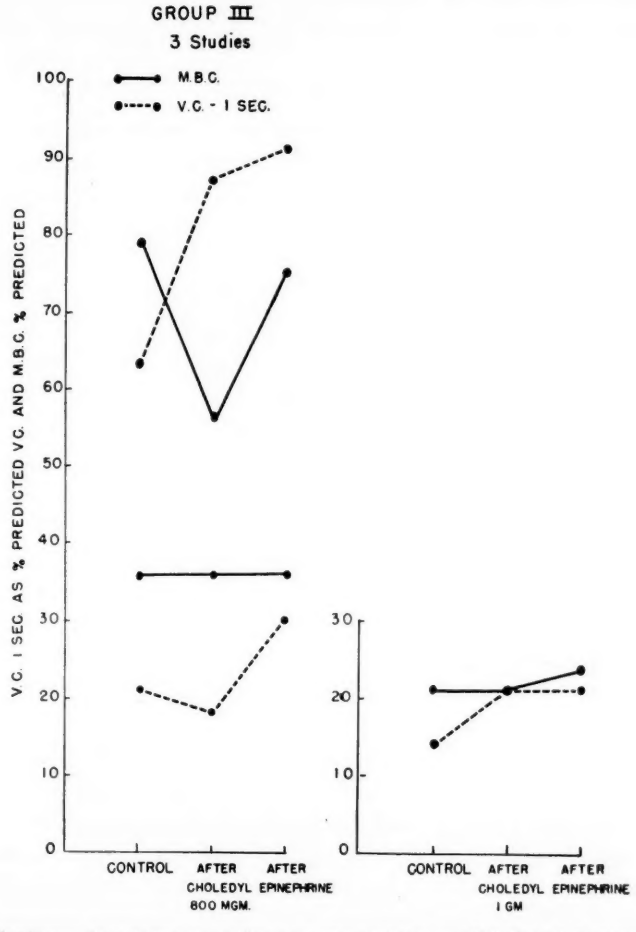

Fig. 2.—Maximum breathing capacity as a percentage predicted and 1 second vital capacity as a percentage predicted vital capacity plotted at the control point, after choline theophyllinate and after epinephrine for 3 cases where the maximum breathing capacity fell or failed to rise at one observation point when the 1 second vital capacity rose.

Conversely in the 3 cases of Group III the 1 second vital capacity demonstrated an antiobstructive effect which was not shown by the maximum breathing capacity.

Lowell and Schiller's[15] studies of changes of the expiratory rate during measurement of the vital capacity in asthma may cast some light on the difference between the maximum breathing capacity and the timed vital capacity in the 9 cases of Group II. They found that during forced expiration

in normal persons air leaves the lung with a speed which changes (i.e., slows) at a constant or almost constant rate. In asthmatics the rate of change in speed of expiration may vary during a single forced expiration. As a result, in some patients a single segment of the vital capacity as measured by the timed vital capacity cannot truly represent the manner in which air leaves the lungs. The maximum breathing capacity being a summation of many breaths in which the patient chooses the rate and depth may well be a more accurate measure of ventilatory function in these cases.

DISCUSSION

Much of our experience substantiates the adverse criticisms which have been leveled against the use of the maximum breathing capacity in bronchial asthma. It has already been pointed out that we have excluded from study certain patients who were too ill to perform the test reliably or whose asthma was made worse by the test. However, in a group of selected patients we have been able to show a high degree of reproducibility of the maximum breathing capacity during the performance of three serial pairs of tests, with fatigue playing a small role. The reproducibility of the 1 second vital capacity in this study was no better than that of the maximum breathing capacity. The 1 second vital capacity has not been found to be a completely satisfactory substitute for the maximum breathing capacity since in one-fourth of the cases it failed to show bronchodilatation when the maximum breathing capacity did. In 8 per cent of the cases the maximum breathing capacity did not show bronchodilatation when the 1 second vital capacity did. In studies where many determinations of ventilatory function repeated at short intervals are necessary, the 1 second vital capacity might well show superiority over the maximum breathing capacity. In short-term studies such as ours the most accurate results are obtained by a combination of both tests of ventilatory function.

One of the problems to be solved in studies of this sort is the establishing of criteria for significant changes of the maximum breathing capacity and 1 second vital capacity in the individual case. In a previous study[1] we considered that all changes in the maximum breathing capacity, which were less than 20 per cent of the control maximum breathing capacity, were not significant. Dann et al.[16] have similarly insisted on a minimum increase of 20 per cent of the control maximum breathing capacity before ascribing significance to the change. This criterion appears to give satisfactory results if one is dealing with severe degrees of airway obstruction. However, in patients with only a moderate degree of airway obstruction, large numerical changes in the maximum breathing capacity may be classed as not significant. For example, in one case studied recently the control maximum breathing capacity was 106 liters per minute with a predicted maximum breathing capacity of 100 liters per minute. Following the administration of a bronchodilator drug, the maximum breathing capacity rose to 123 liters per minute. This was an increase of only 16 per cent over the control level, but, in view of the reproduci-

bility of duplicate maximum breathing capacities, surely this increase was significant. Comroe[17] has stated that he believes rises of 10 liters per minute in the maximum breathing capacity to be significant. While we have no final answer to this problem analysis of our studies indicates that significance can be ascribed to rises of 20 per cent in the maximum breathing capacity when the control maximum breathing capacity is less than 50 liters per minute, and that rises of 10 liters per minute can be considered significant when the maximum breathing capacity is greater than 50 liters per minute. It is also helpful to express the degree of improvement after the test drug as a percentage of the improvement occurring after the known bronchodilator. For example, in a case where the control maximum breathing capacity was 45 liters per minute, and after the test drug the maximum breathing capacity rises to 60 liters per minute, and after epinephrine to 75 liters per minute, the drug would have 50 per cent of the bronchodilating effect of epinephrine under these conditions of study. There is the possibility that in a study of this sort the administration of the test drug may have altered the patient's response to epinephrine. A study of this point is in progress.

SUMMARY AND CONCLUSIONS

The pathophysiology of bronchial asthma is briefly reviewed, and the shortcomings of clinical methods in the evaluation of drugs for its treatment are noted. A method is described whereby the maximum breathing capacity test and the timed vital capacity determination are used to compare the bronchodilating effects of a test drug and a known bronchodilator drug in asthmatic patients.

The relative value of the maximum breathing capacity test and the 1 second vital capacity in evaluating bronchodilatation are assessed. We have found that the maximum breathing capacity test aggravates asthma in some cases, and that in severely ill patients reliable results cannot be obtained. However, in a selected group of patients the maximum breathing capacity was found to be highly reproducible with duplicate determinations showing a mean variability of 2.3 ± 2.3 liters per minute or 4.7 per cent of determined maximum breathing capacity. The variability was significantly smaller when the maximum breathing capacity was low than when it was high. One second vital capacities performed "simultaneously" with the maximum breathing capacities showed about the same degree of variability of duplicate determinations as the maximum breathing capacity with a mean difference between duplicates of 48 ± 60 c.c. or 5.2 per cent determined 1 second vital capacity.

The correlation between 1 second vital capacity and maximum breathing capacity was very high. In one-fourth of our studies, however, there was at least one observation point where the 1 second vital capacity failed to demonstrate bronchodilatation when the maximum breathing capacity did. The converse was true in 8.5 per cent of cases. In this study, the 1 second vital capacity did not appear to be superior to the maximum breathing capacity. The most accurate results were obtained by a combination of both methods.

Criteria are suggested for the evaluation of the significance of changes in maximum breathing capacity after the administration of test drugs.

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THE ORIGIN OF PLASMA CHOLESTEROL AND THE RATES OF EQUILIBRATION OF LIVER, PLASMA, AND ERYTHROCYTE CHOLESTEROL

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WHEN C^{14}-acetate is administered to an animal, cholesterol-C^{14} appears rapidly in plasma. By comparing the amount appearing in hepatectomized and in control, sham-operated dogs, it has been shown that practically all the plasma cholesterol of endogenous origin is derived from liver. This was reported in preliminary form in 1951.[1] The same conclusion regarding the origin of plasma cholesterol in rats was reached simultaneously by Friedman, Byers, and Michaelis,[2] who compared the rates at which the plasma cholesterol levels returned to normal in partially hepatectomized and normal rats, rendered hypocholesterolemic by plasmapheresis.

The present paper gives the details of the experiments on hepatectomized dogs, including evidence for extrahepatic synthesis of cholesterol and fatty acids from C^{14}-acetate in a number of tissues. In addition, results are presented of a study of the rates of appearance of newly synthesized cholesterol molecules in the free and esterified fractions of liver, plasma, and erythrocytes in dogs. This investigation of the kinetics of liver and blood cholesterol was suggested by the observation that by 4 hours after the administration of C^{14}-acetate to normal dogs, the free cholesterol-C^{14} was distributed almost equally between the free liver and blood cholesterol. Such a rapid rate of transfer from liver to plasma is not compatible with the reported turnover time of plasma cholesterol of about 12 days,[3] and suggests the possibility that the free cholesterol molecules in liver and plasma interchange so rapidly that liver and plasma may be considered from this standpoint as a single compartment or entity. The conclusions of this study have been previously reported and their significance discussed.[3, 6]

Harper, Neal, and Hlavacek[7] have reported the results of a similar investigation; they used much larger doses of C^{14}-acetate and did not study erythrocytes.

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EXPERIMENTAL

C^{14}-Acetate.—A 0.1 M solution of carboxyl-labeled sodium acetate containing 100 μc per mM was used in all experiments, except where otherwise noted.

Hepatectomy Procedure.—Large dogs were hepatectomized by a one-stage procedure consisting of forming an Eck fistula by a side-to-side anastomosis of the inferior vena cava and the portal vein, double ligation and section of the portal vein and hepatic artery, immediately followed by excision of the liver, lobe by lobe. It was possible to show by subsequent analysis that the small amount of residual liver tissue was almost nonfunctional during the experiment. Whole blood was given to replace that lost during the surgical procedure. Shortly after completion of the the surgery, the dog was moved into a hood, and C^{14}-acetate was administered intravenously. Glucose solution was given at a rate of approximately 0.25 Gm. per kilogram of body weight per hour. In certain experiments respiratory CO_2 samples were taken at intervals and the specific activity determined as $BaCO_3$.

The experiments were terminated 4 or 6 hours after acetate administration by exsanguination of the anesthetized animal, and total cholesterol and fatty acids were obtained from plasma and from a number of tissues.

The hepatic vena cava of each hepatectomized dog with adherent residual liver tissue was worked up in the same way as liver to give an idea of the amount of liver tissue left and its degree of function. This fraction is referred to in the tables as "liver residue."

In each experiment two dogs of as nearly the same weight as possible were used, one being hepatectomized and the other usually sham-operated. Acetate of the same specific activity was given in a definite amount per kilogram of body weight to each; hence, the results for the two dogs in each experiment are comparable. Some details of the individual experiments follow:

Experiment 1.—The hepatectomized dog (wt. 9.0 kg.) was prepared under Nembutal anesthesia and given 20 μc of acetate per kilogram of body weight intravenously in a single dose. The sham-operated control dog (wt. 7.3 kg.) was opened, the portal vein and vena cava were exposed, but the anastomosis was not carried out. The gall bladder was emptied by aspiration, the common duct ligated and the abdomen closed. Ten microcuries of acetate per kilogram were given intravenously. Respiratory CO_2 samples were collected from both animals and they were both sacrificed 4 hours after acetate administration.

Experiment 2.—Ether anesthesia was used for the hepatectomy; the dog recovered consciousness shortly after completion of the surgery and before the acetate dose (40 μc per kilogram) was given. The control dog was sacrificed 1.25 hours after the acetate administration.

Experiment 3.—The control dog was subjected to anastomosis of the vena cava and the portal vein. A mesenteric thrombosis developed and the dog died 3 hours after injection of the C^{14}-acetate. In spite of the lack of knowledge as to when the blood supply to the intestine was cut off, the results on this animal are included.

Unanesthetized Dog Experiments.—C^{14}-acetate was given to a series of normal dogs by stomach tube, 16 to 20 hours after the last feeding. A dose of 10 μc per kilogram of body weight was used, and the stomach tube rinsed with about 50 ml. of water. Blood samples were taken at 1, 2, 4, 8, 24, and 48 hours in heparinized syringes, and immediately centrifuged. The plasma and red blood cell volumes were measured and they were worked up as described below.

Three litter-mate dogs were studied to investigate the difference between oral and intravenous administration on the efficiency of utilization of C^{14}-acetate, for blood cholesterol synthesis. Each dog was given both an oral and an intravenous dose at an interval of several weeks. Blood samples were taken at 4 and 24 hours after the first dose, and just before, 4 and 24 hours after the second dose.

Serial Liver Biopsy Procedure.—Dogs were anesthetized with Nembutal and a tracheal cannula was inserted. This was connected to a T-tube with flutter valves on either side of the T. The intake was left open and the outlet was passed through a shallow layer of NaOH solution and then conducted out of the window. This arrangement prevented any $C^{14}O_2$ from

escaping into the room. The liver was exposed and the C^{14}-acetate injected intravenously into a mesenteric vein, a leg vein, or given by stomach tube. Samples of liver were removed at various intervals from 5 minutes to 7 hours subsequently. Each 2 to 5 Gm. sample was taken from a different lobe and bleeding was controlled by the following procedure: A series of overlapping, interrupted mattress sutures was laid across the end of a lobe (the deeper lying lobes being used first), using heavy cotton or silk sutures and as small a needle as possible. After tying the sutures, the distal part of the lobe was cut off with a sharp knife and the surface cauterized with coagulation current. Gelfoam was applied over the surface and the lobe replaced in the abdominal cavity only after it had been observed to remain dry for some time. The opening was filled with saline packs until time for the next biopsy. Hemorrhage was readily controlled by this method and there was no operative fatality. In all cases in which sterile procedures were employed, the dogs survived indefinitely. Blood samples were taken at intervals usually at the same time as the liver samples. No blood or other fluids were administered.

Isolation and Assay of Cholesterol.—Tissues from hepatectomized and control animals were saponified in alcoholic alkali and total cholesterol isolated and assayed as previously described.[8]

In all other experiments, when only total cholesterol was wanted, the tissue or blood sample was saponified directly and the nonsaponifiable fraction obtained; when both free and total or free and ester values were desired, a total lipid extract was prepared using 3:1 alcohol-ether mixture for red cells or whole blood and a 1:1 alcohol-acetate mixture for other samples. Analysis for free and total cholesterol was carried out as described by Sperry and Webb[9] and free cholesterol digitonide prepared for assay by essentially the same procedure, except that only a slight excess of digitonin was added. Total cholesterol was obtained by saponification of a separate aliquot and in most cases the nonsaponifiable fraction was isolated by extraction with petroleum ether. This procedure served as a check on the possibility of contamination of the free cholesterol digitonide by high specific activity substances present in the total lipid extract. Esterified cholesterol digitonide was obtained by evaporation to dryness of the filtrate from the free cholesterol digitonide, and complete saponification as described above.

Fatty acids were obtained as the total fatty acid fraction after saponification of the tissue, as previously described.[8]

RESULTS

Effect of Hepatectomy.—The data in Table I for plasma cholesterol show that hepatectomy decreased the rate of appearance of cholesterol-C^{14} to a very small fraction of the control rate in the two experiments in which the C^{14}-acetate was given intravenously. The observed counting rates for the plasma cholesterol samples from the hepatectomized dogs were 11 and 12 c.p.m. which is just barely significant; whereas the control animals gave values of 362 and 178. This last value was for blood taken at 1.25 hours and would have been higher at 4 hours.

Although the ligation of the common duct carried out in Experiment 2A (the control animal) would eventually affect the rate of hepatic cholesterol synthesis, there is no reason to believe that it would have an appreciable effect until the gall bladder becomes distended. Cholesterol synthesis from C^{14}-acetate in liver is completed within an hour after injection as shown in Fig. 2, and consequently ligation of the common duct may be considered to be without significant effect on the results in this experiment.

In Experiment 3 B, the acetate was injected into the lumen of the intestine; evidence from other experiments suggests that the absorption is very slow in

anesthetized animals and hence acetate of high specific activity (S.A.) presumably remained in contact with the intestinal mucosa for some time. Intestinal mucosa cholesterol had an exceedingly high S.A., about 100 times as high as plasma. However, the plasma value was definitely higher than in Experiments 1 and 2,

TABLE I. SPECIFIC ACTIVITIES (S.A.) OF CHOLESTEROL FROM PLASMA AND TISSUES OF CONTROL AND HEPATECTOMIZED DOGS AFTER INJECTION OF C^{14}-ACETATE

	CONTROL DOGS			HEPATECTOMIZED DOGS		
EXPERIMENT NO.	1 A	2 A	3 A	1 B	2 B	3 B
DURATION IN HOURS	4	1.25	3†	4	4	4
ROUTE OF ADMINISTRATION	I.V.	I.V.	JEJUNUM	I.V.	I.V.	JEJUNUM
SPECIFIC ACTIVITY OF ISOLATED CHOLESTEROL IN μC PER GM. OF CHOLESTEROL						
Plasma	0.414	0.311	0.062	0.0006*	0.0060	0.018*
Liver	0.418	0.463	0.266	—	—	—
Liver residue	—	—	—	0.0006*	0.0052	0.0087
Bile	0.532	—	0.092			
Small intestine, whole	—	0.062	—	—	0.076	
Small intestine, mucosa	0.107	—	0.058	0.0124	—	2.14
Small intestine, wall	0.065	—		0.0060*		0.432
Lungs	0.078	0.022		0.014	0.0052	0.0143
Skin	0.0091	—		0.0064		0.0373
Kidney		0.024		0.100	0.071	0.016
Diaphragm	0.045	0.021		0.011	0.002*	0.002*
Spleen	—	0.079		0.0008*	0.0048	0.017
Adrenals	—	0.021		0.0006*	0	0.002*
Aorta	—	0.022		0.0033*	0	0.0050
Sternum				0.0066*	—	0.025
Fat depot				0.0027*	0.0004*	
Heart		0.027			0.0004*	
Red cells		0.17			0.0004*	
Skeletal muscle					0.0027*	
Pancreas					0.0014*	
Stomach					0.0263*	

*Observed counting rate was less than 10 c.p.m. above background so the value is not considered to be significantly different from zero. All S.A. values are calculated from observed counting rates by correcting to infinitely thick sample values by means of a factor in the range 1.0 to 1.5, then calculated to a standard dose of 10 μc per kg. body weight, and finally multiplied by 1.731×10^{-3} to give μc per Gm. of cholesterol. Thus, 0.01 μc per Gm. corresponds to about 20 to 80 c.p.m. above a background of 27 ± 2, depending on dose.

†Dog died during the experimental period as a result of portal thrombosis. Although abnormal in certain respects, since the portal occlusion had produced an intense cyanosis of all the mesenteric circulation, the liver cholesterol S.A. value showed that a considerable fraction of acetate had been absorbed and metabolized and the results are therefore included.

TABLE II. SPECIFIC ACTIVITIES OF THE TOTAL FATTY ACID FRACTION FROM TISSUES OF CONTROL AND HEPATECTOMIZED DOGS

	CONTROL DOGS		HEPATECTOMIZED DOGS		
EXPERIMENT NO.	1 A	2 A	1 B	2 B	3 B
Plasma	0.0384	0.0317	0.0051		0.185
Liver	0.160	0.0671	—	—	—
Liver residue	—	—	0.0024*	0.0189	0.008
Intestinal mucosa			0.0672		
Intestinal wall	0.0789		0.0789		1.689†
Adrenal	0.0346		0.0951	0.044	0.199
Aorta			0.0306	0.039	0.053
Spleen		0.040	0.0091	0.060	0.0104
Lung	0.045	0.092	0.0682	0.0698	0.067
Kidney		0.101	0.0794	0.162	0.104
Diaphragm		0.0024*	0.027	0.040	0.109
Skin		0.004*	0.0085		0.0016*

All values are expressed in μc per gram, calculated for a standard dose of 10 μc per kilogram and are comparable with figures in Table I for cholesterol.

*Observed counting rate was less than 10 c.p.m. above background so that value is not considered to be significantly different from zero.

†This dog was given acetate intrajejunally and the intestinal fatty acids, like cholesterol, had extremely high S.A. values.

suggesting that traces of cholesterol synthesized by the intestinal mucosa may appear in plasma within 6 hours even under the unfavorable conditions of anesthesia. No definite conclusions can be drawn from the control animal in this experiment because the mesenteric thrombosis, which developed as a result of the operative procedure at an unknown time, resulted in a complete stoppage of the blood supply to the intestine.

The mean value for all three controls was 32 times as high as the mean for all the hepatectomized dogs, indicating that the rate of appearance of newly synthesized cholesterol in plasma was decreased to not more than a few per cent of the normal value by removal of the liver. Evidence that oxidative metabolism of acetate in extrahepatic tissues was not greatly changed by hepatectomy is shown in Fig. 1, which gives the S.A. of the respiratory CO_2 during the whole experimental period in one experiment. Both animals were able to metabolize

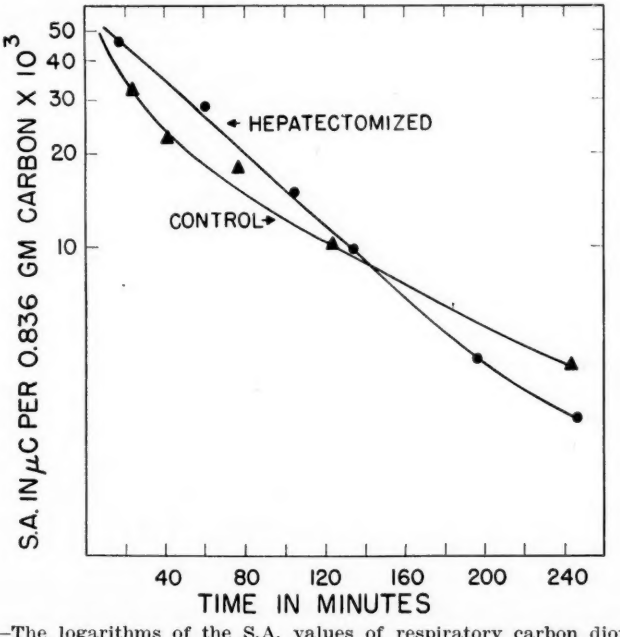

Fig. 1.—The logarithms of the S.A. values of respiratory carbon dioxide for the control and hepatectomized dogs in Experiment 1, plotted against time. The S.A. is expressed in microcuries per 0.839 gram of carbon to make it directly comparable to microcuries per gram of cholesterol, used in subsequent figures. The values are also calculated to a standard dose of 10 μc per kilogram of body weight.

acetate at essentially normal rates. Furthermore, the presence of isotopic carbon in both cholesterol and fatty acids in certain extrahepatic tissues, shown in Tables I and II, suggests that the difference in the plasma cholesterol values was not due to an inability on the part of the hepatectomized dogs to utilize the isotopic acetate for synthetic purposes.

Extrahepatic Synthesis of Cholesterol and Fatty Acids.—It is not known whether intravenously injected acetate is uniformly distributed and uniformly diluted with endogenous acetate or not, so the S.A. values for cholesterol in extrahepatic tissues of the hepatectomized dogs cannot be taken as strictly proportional to rates of regeneration of tissue cholesterol, but they do indicate which

tissues are capable of cholesterol synthesis under these conditions and give an idea of the relative rates. Intestine, kidney, lung, skin, and probably stomach appear to synthesize cholesterol to some extent since they all contained cholesterol with significantly higher isotope content than the plasma of the same animal. It will be noted that the S.A. of cholesterol of all tissues in hepatectomized dogs tended to be lower than the corresponding tissues in controls; this was probably due to the rapidity of appearance of plasma cholesterol molecules in many tissues,[3] but possibly also to indirect effects of hepatectomy on cholesterol synthesis in extrahepatic tissues. The control animal in Experiment 2 was sacrificed at 1.25 hours to decrease the effect of transport of hepatic cholesterol as much as possible. It will be noted that the liver S.A. is as high as in Experiment 1, but that plasma and other tissues are relatively lower.

Table II gives the S.A. values for total fatty acid fractions from some of the same tissues. Although only preliminary in nature, these data bring out several points of interest. Endogenous plasma fatty acids do *not* appear to originate entirely in liver, as shown by the high plasma fatty acid S.A. value in the hepatectomized dog, 3 B. Since this value is considerably higher than that of any other tissue studied except intestine, it may be tentatively concluded that some of the fatty acids synthesized by the intestine appear rapidly in plasma. Chaikoff and associates[10, 11] have shown that plasma phospholipids originate only in liver; so the activity observed in this experiment was presumably due to fatty acids of neutral fat or cholesterol esters or both. The results of Experiment 1 suggest that the major fraction of endogenous plasma fatty acids do, however, come from liver.

Fatty acid synthesis from acetate in adrenal, intestine, lung, kidney, aorta, and probably spleen is indicated by the values for these tissues in the hepatectomized dog of Experiment 1. Masoro and associates[12] have previously reported fatty acid synthesis in extrahepatic tissues of rats from C^{14} glucose, and Popjak and Beeckmanns[13] with the aid of both D_2O and C^{14}-acetate, concluded that synthesis of fatty acids occurs in situ in various tissues in rabbits.

TABLE III. RELATIVE RATES OF SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS IN VARIOUS TISSUES OF CONTROL AND HEPATECTOMIZED DOGS

	CONTROLS		HEPATECTOMIZED	
TISSUE	CHOLESTEROL μC	FATTY ACIDS μC	CHOLESTEROL μC	FATTY ACIDS μC
Plasma	4.5	0.61	0.060	0.28*
Liver	12.4	22.8	—	—
Liver residue	—	—	0.14	5.0
Intestine	3.76	9.85	0.59	44.6
Lung	2.4	12.8	0.34	6.18
Spleen	3.43	4.67	0.19	7.2
Adrenal	6.22			43.9
Aorta	0.32			7.41

Values are expressed in μc per 100 gram of tissue for a standard dose of 10 μc per kilogram × 100, and were calculated from the mean values for all dogs that were given intravenous acetate. These values are proportional to the rates of synthesis.

*Based on an assumed value for fatty acid concentration in plasma of 250 mg. per cent since actual value was not determined.

Table III gives the relative rates of synthesis of cholesterol and fatty acids in a number of tissues expressed in microcuries in the cholesterol (or total fatty acids) present in 100 Gm. of tissue. These values represent the fraction of the

dose given which was incorporated in this amount of material, and they are proportional to the rates of synthesis in milligrams per 100 Gm. of tissue per hour. They are included to show the greater rate of synthesis of fatty acids than of cholesterol even in tissues in which the turnover time for cholesterol was faster than for fatty acids. Although comparisons between different tissues may be somewhat affected by differences in distribution and dilution of C^{14}-acetate, this should not affect comparisons between cholesterol and fatty acid synthesis in any one tissue.

Rates of Appearance of Cholesterol-C^{14} in Plasma Free, Plasma Esterified and Erythrocyte Fractions.—The maximum S.A. of whole blood cholesterol was reached very rapidly, after either oral or intravenous administration of C^{14}-acetate, usually in 2 to 4 hours. It then fell much more slowly than it had risen, eventually reaching a rate of decrease of 50 per cent in about 8 days. This rate was maintained from about the third day for about two weeks and the S.A. curve then gradually flattened out.

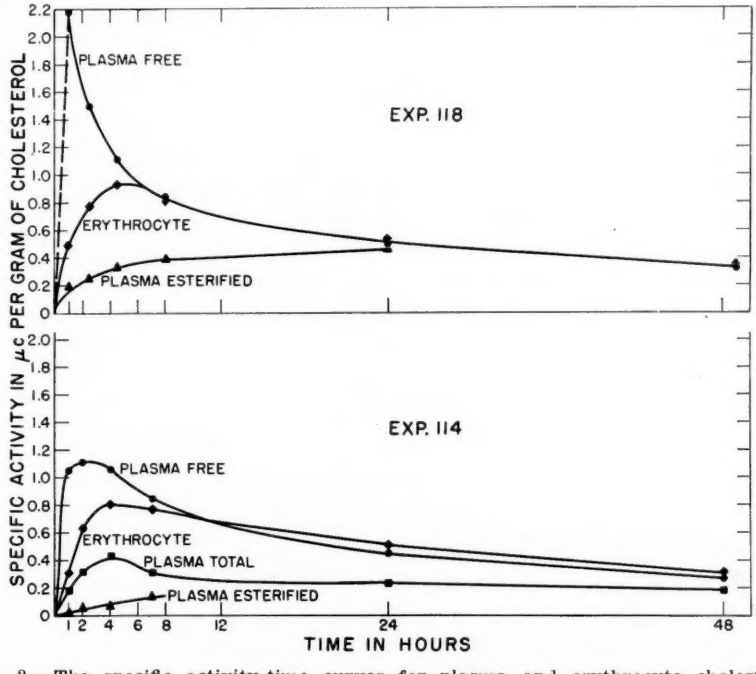

Fig. 2.—The specific activity-time curves for plasma and erythrocyte cholesterol fractions for two experiments on normal, unanesthetized dogs given 10 μc of 1-C^{14}-acetate by stomach tube.

The three individual components of blood cholesterol, plasma free, plasma esterified, and erythrocyte free fractions differed greatly in the rapidity with which they acquired labeled, newly synthesized cholesterol molecules, as shown in Fig. 2. Plasma free cholesterol reached a maximum value in 1 to 2 hours; erythrocyte-free in 3 to 8 hours; and plasma esterified only after about 2 days. All three gradually approached approximately the same value.

In unanesthetized dogs, the time at which the plasma free cholesterol S.A. attained peak values was not appreciably affected whether the acetate was

administered orally or intravenously. However, the maximum value attained was much greater following oral than intravenous administration, not only for the plasma free fraction, but for both other fractions as well (Table IV).

TABLE IV. EFFECT OF THE ROUTE OF ADMINISTRATION OF ISOTOPIC ACETATE ON THE MAXIMUM SPECIFIC ACTIVITY OF BLOOD CHOLESTEROL

EXP. NO.	DOG	ROUTE OF ADMINISTRATION	SPECIFIC ACTIVITY OF CHOLESTEROL IN μC/GM. FOR 10 μC/KG. DOSE		
			PLASMA FREE	PLASMA TOTAL	RED CELL
1	A	Oral	0.515	0.270	0.602
	B	Oral	0.540	0.208	0.398
	C	Intravenous	0.166	0.075	0.108
2	C	Oral	1.154	0.610	0.979
	B	Intravenous	0.133	0.104	0.112
	A	Intravenous	0.125	0.0540	0.033

Each dog was given two doses of 10 μc per kilogram of body weight of 1-C^{14}-sodium acetate, one dose by stomach tube and the other by intravenous injection. The second experiment was done about 1 month after the first; the acetate specific activity was 500 μc per millimole in the first experiment and 100 μc per millimole in the second. Blood samples were taken at 4 and 24 hours after acetate administration in both experiments. The effect of residual cholesterol-C^{14} in the blood at the beginning of the second experiment was corrected for by taking a blood sample just before giving the C^{14}-acetate and subtracting these S.A. values from the subsequent ones.

The S.A. values depend also on the S.A. of the administered acetate, as Harper, Neal, and Hlavacek[7] reported.

Serial Liver Biopsy Results.—The rapidity with which cholesterol synthesis from a small dose of injected acetate is completed in liver is shown for 4 of the 7 experiments carried out in Fig. 3.

Liver free cholesterol is obviously the first product of synthesis. Its specific activity reached a peak value in less than an hour in every case and in some experiments apparently at about 15 minutes after injection of acetate into a mesenteric vein. These results are in agreement with those reported for liver cholesterol in mice by Beeckmanns and de Elliott,[14] who found the maximum S.A. at 30 minutes. The rapid decrease in S.A. after the time of maximum value was due to rapid interchange of liver cholesterol molecules with unlabeled molecules in the plasma free fraction, and more slowly with those in red cells, plasma esterified fraction, and with the cholesterol in many tissues, particularly spleen and other viscera.

The small differences between the S.A. values of free and total cholesterol fractions of liver reflect the small amount of ester present in normal dog liver. However, in one experiment esterified cholesterol was isolated. (The values are shown in Fig. 3.) It appears that the esterified cholesterol in liver rapidly acquired a considerable amount of the newly synthesized cholesterol, but that it maintained a definitely lower S.A. than the free form for some time.

In two experiments the isotopic acetate was given by stomach tube to the anesthetized dogs. In one experiment there was a lag of about an hour followed by essentially the same values as in Fig. 2. In the second experiment, all liver and blood cholesterol S.A. values were below 150 c.p.m. during the first three hours, but the blood value 26 hours later was over 2,000 c.p.m., which was in the expected range for the dose given. These results suggest that the anesthetic inhibited absorption of the acetate.

The average time for the peak S.A. of liver free cholesterol was 40 minutes (five animals), and for plasma free cholesterol, 85 minutes, using an acetate dose of 0.1 mM per kilogram of body weight. Harper, Neal, and Hlavacek,[7] using 30 times the acetate dose, found peak values at 60 minutes for liver free and 120 minutes for plasma free cholesterol.

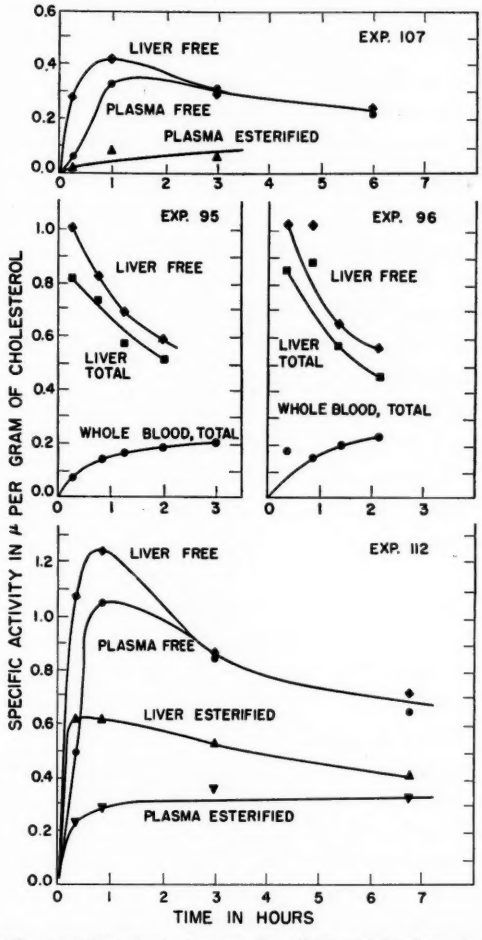

Fig. 3.—The specific activity-time curves for liver and plasma cholesterol fractions for four experiments on dogs given intravenous 1-C^{14}-acetate. In Experiments 95, 96, and 112 acetate was injected into a mesenteric vein and in Experiment 107 into a leg vein; this difference probably accounts for the lower specific activities resulting in Experiment 107.

DISCUSSION AND CALCULATIONS

The rates of movement of isotopically labeled cholesterol molecules from liver to plasma may be roughly estimated graphically for the free form from the data in Fig. 3.

The expressions for the specific activity of a labeled molecule in both compartments of a two-compartment system during mixing is given by:

$$a = (a_o - C)\ e^{-kt} + C, \text{ and } b = C(1 - e^{-kt})$$

where a is the S.A. in the first compartment and b that in the second, C is the

S.A. of the system at equilibrium, and a_o the value of a at t = O. It follows that $(a - b) = a_o e^{-kt}$, and the log of (a – b) plotted against time will give a straight line from which the value of the rate constant k may be estimated. In the present instance, C is not a constant but decreases slowly with time, and, furthermore, when t is O, a is O rather than the maximum value a_o. However, it can be seen that liver free cholesterol S.A. reached a peak value very rapidly and that the difference between the specific activities of the liver free and plasma free cholesterol fractions approached zero rapidly in relation to the rate of decrease of the equilibrium value. When the log of this difference is plotted against time, a straight line is obtained, as shown in Fig. 4 for two of the serial liver biopsy experiments. The half equilibration time was estimated as 17 minutes for Experiment 107, and 20 minutes for Experiment 112. In view of the other processes occurring simultaneously, these values should be taken as rough approximations. The close approach to linearity of the data in Fig. 4 suggests a first order process during the period under consideration thus supporting the assumption that the mixing of the labeled molecules in these two compartments is the dominating process during this time and is largely responsible for the decrease in specific activity of liver free cholesterol.

The second process which occurs, evident from Fig. 2, is the interchange of plasma free and red cell cholesterol molecules. Since the latter are virtually all free, no esterification is involved. This process was also found to approximate first order reaction kinetics. Fig. 5 shows the log of the difference between plasma free and red cell free cholesterol S.A. values for 2 typical experiments. The half equilibration times for 4 experiments, one serial liver biopsy and three normal unanesthetized dogs, were estimated to be 1.83, 1.75, 1.05, and 1.10, respectively, giving a mean of 1.43 hours. This rate is slightly slower than that previously reported for this process in vitro of about 1.10 hours.[15] Since there were fewer variables involved in the in vitro studies, the faster value is considered more nearly accurate. This equilibration is actually between the free cholesterol of both plasma and liver on the one hand and that of red cells on the other, as is evident from the fact that liver free and plasma free cholesterol S.A. values are equal during most of the time, and also because the S.A. of plasma free does not fall nearly as much as in the in vitro studies. The total amount of plasma free cholesterol is relatively small so that during equilibration with red cell cholesterol, its S.A. would necessarily fall to a very low value if the labeled molecules were not being replaced from the larger reservoir in the liver. This may explain the slower rate observed since it is essentially an over-all rate for both processes.

It is of interest that Harper and associates[7] observed a rapid decrease in S.A. of plasma free cholesterol following hepatectomy. They reported a "turnover time" of roughly 1.5 hours between plasma free cholesterol and an unidentified "reservoir of untagged cholesterol." This can be explained as due to equilibration between plasma free and red cell cholesterol, which in the absence of the liver would be expected to have a half time of about 1.10 hours, as in the in vitro studies, corresponding to an equilibration time of 1.58 hours.

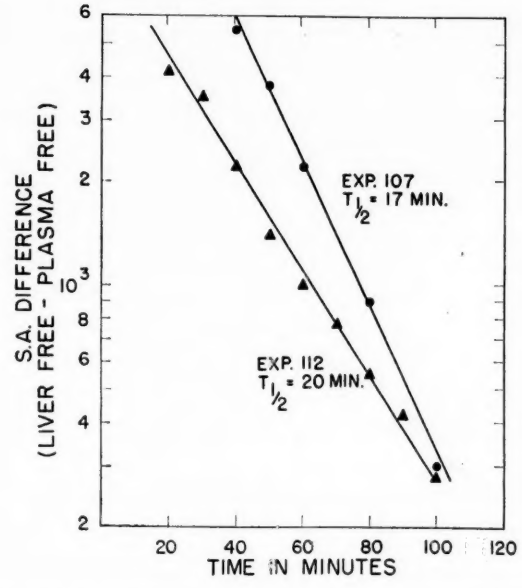

Fig. 4.—The rate of equilibration of free cholesterol between liver and plasma. The difference between the specific activity values of liver free and plasma free cholesterol, plotted on a logarithmic scale against time for two experiments. The apparently linear character of the relationship permits estimation of the rate of this equilibration process from the slopes to be approximately 17 and 20 minutes. The points represent equal time intervals taken from the smoothed curves of Fig. 3.

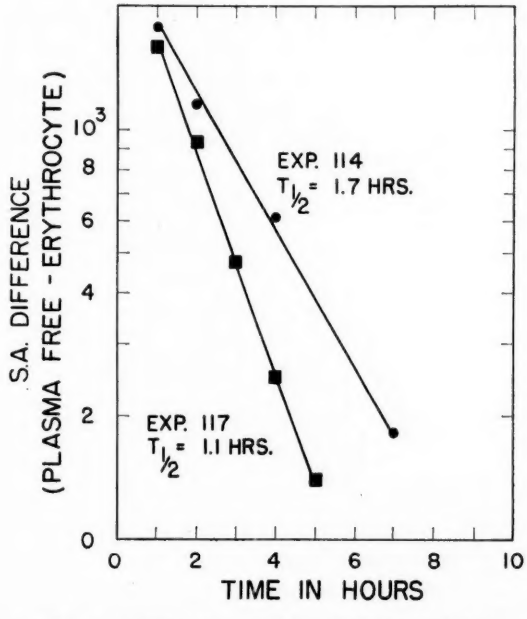

Fig. 5.—The rate of equilibration of free cholesterol between plasma and erythrocytes. The difference between the specific activity values of plasma free and erythrocyte free cholesterol fractions, plotted on a logarithmic scale against time for two typical experiments. The apparently linear character of the relationship permits estimation of the rate of this process from the slope of the lines. The mean value for four experiments was 1.43 hours.

The third process is the appearance of labeled cholesterol molecules in the plasma esterified fraction. This was found to be considerably slower, and also appeared to be somewhat more variable. The average value for plasma free plus red cell (i.e., whole blood free cholesterol S.A.) was calculated from the individual S.A. values and the concentrations, and the rate of equilibration of whole blood free cholesterol with the esterified fraction was estimated graphically to be about 50 per cent in 7 hours from the data shown in Fig. 2, for Experiment 118. In most of the other experiments, the plasma esterified S.A. values were not determined directly but were calculated from the free and total S.A. values and concentrations and were consequently less reliable. However, it has been repeatedly observed that the plasma free (and erythrocyte) cholesterol S.A. was significantly higher than the plasma total value 24 hours after the administration of acetate but not 48 hours after. This observation is in accord with a half equilibration time of 7 hours which corresponds to 91 per cent equilibration in 24 hours and 99 per cent in 48 hours.

The fraction of isotope given as acetate which was converted into cholesterol in liver may be estimated from the total amount present in liver and blood at the time of maximum specific activity. Total blood volume was taken as 6 per cent of body weight and liver weight as 3 per cent. In three experiments in which the acetate was given by injection into a mesenteric vein, the average yield was 1.1 per cent.

The total amount of isotope in cholesterol present in the liver-blood system did not change as rapidly with time as any of the individual components; in Experiment 103, for example, only a 25 per cent decrease occurred between 2 and 6 hours. This decrease was probably very largely due to movement of plasma cholesterol molecules into body tissues, particularly into spleen and other viscera, but it is in small part due to metabolic breakdown of cholesterol in the liver and excretion in bile.

Such rapid interchanges of molecules between tissues in which their concentrations are quite different, with no net changes in concentration being produced, must be considered in relation to the fact that cholesterol is a water-insoluble molecule and is normally present bound to specific proteins. The molecules are hence not readily diffusible in the same sense that water-soluble molecules are, but the distribution between tissues must be partly determined by the amounts of specific proteins capable of binding cholesterol and their relative affinities for it.

SUMMARY

1. Removal of the liver in dogs prevents almost completely the appearance of C^{14}-cholesterol in plasma subsequent to the injection of C^{14}-acetate, as compared with control sham-operated dogs.

2. Hepatectomized dogs metabolized C^{14}-acetate to $C^{14}O_2$ at essentially the same rate as controls, and cholesterol synthesis occurred to a significant extent in a number of extrahepatic tissues, especially intestine, kidney, lung, and skin.

3. Fatty acid synthesis also was demonstrable in all tissues examined in both control and hepatectomized animals.

4. Serial liver biopsies following injection of C^{14}-acetate showed that the peak value for isotope concentration in liver free cholesterol was reached in about 40 minutes. The free cholesterol in liver had a higher specific activity than the esterified.

5. The rate of appearance of isotopically labeled (and hence newly synthesized) cholesterol in the blood of normal, intact dogs given oral C^{14}-acetate was most rapid in the plasma free fraction (peak in about 1 hour), next in the red cell fraction (peak in 3 to 8 hours) and least rapid in plasma esterified fraction.

6. The specific activities of cholesterol in all the previously mentioned fractions approach approximately the same value at such a rapid rate that the equilibration must be due to an interchange of molecules.

7. Rates of equilibration, expressed as half-times, were estimated to be about 20 minutes for the liver free–plasma free system, 1.4 hours for the plasma free–red cell system, and about 7 hours for whole blood free–plasma esterified system.

8. About 1 per cent of the dose of C^{14}-acetate injected into the mesenteric circulation was converted into liver-blood cholesterol.

9. Orally administered acetate resulted in considerably higher values of blood cholesterol S.A. than injection into veins, except those draining into the portal system.

We take pleasure in acknowledging the assistance of Adele Leopardi and Velta Slesers, and the mathematical advice and assistance of Dr. Ernest C. Anderson.

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THE USE OF C^{14}-LABELED ACETATE TO STUDY CHOLESTEROL METABOLISM IN MAN

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THE studies described in this report were undertaken, first, to determine the rate at which cholesterol-C^{14} appeared in the plasma after the administration of carbon-14-labeled acetate to man; and second, to determine the relationship between the dose of acetate and the specific activity of the labeled cholesterol. The role of acetate in the biosynthesis of cholesterol is well known, and specific activity-time curves have been reported for cholesterol-C^{14} in the plasma of dogs[1-3] following single doses of the precursor acetate. Hellman, Rosenfeld, and Gallagher[4] fed 2-C^{14}-acetate to six patients and followed the buildup and the decline of radiocarbon activity in the plasma free and the ester cholesterol over the period, 8 hours to 5 weeks. No studies have been reported that emphasize the changes that occur during the first 24 hours in human subjects. This paper describes this period in some detail. The following topics are discussed: (a) the development of convenient and accurate methods for the radioassay of C^{14}-labeled cholesterol in plasma and tissues; (b) the peak of the specific activity-time curve, and the influence of the route of administration and other factors on the shape of the curve; and (c) the dose of C^{14}-acetate required to provide suitable radioactivity for counting cholesterol and its derivatives in plasma, tissues, and in urinary corticoids.

METHODS

A. *The Tracer Dose.*—All the studies in this report were performed using 100 μc or 200 μc of carboxyl-labeled sodium acetate (1-C^{14}-acetate). The acetate was given either orally or intravenously, and in some cases doses were given by each route. Carboxyl-labeled acetate† with a specific activity of 1.0 mc. per millimole was used. The individual doses (100 μc) contained 8.2 mg. of sodium acetate, and no additional carrier was added. Oral doses were diluted with water to a convenient volume for administration. Intravenous doses were prepared by diluting the acetate to a suitable volume with physiologic solution of sodium chloride. This solution can be sterilized without any effect on the acetate.

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*Los Alamos Scientific Laboratory.

**Argonne Cancer Research Hospital.

†Procured from Tracerlab by authorization of the Isotopes Division, USAEC, under the University of Chicago Clinics' General Authorization for Human Use, and approved by the Clinics' Radioisotope Committee.

The dose of 100 μc was chosen so that repeated doses could be given to the same subject without exceeding accepted values for the maximum permissible dose for man. Our studies of $C^{14}O_2$ in expired air after the administration of 1-C^{14}-acetate demonstrated that approximately 56 per cent of the radiocarbon was eliminated during the first 24 hours.* On this basis, we have made the assumption that a single 100 μc dose will result in the "retention" of not more than 25 μc of C^{14} in the slowly exchanging "fat compartments" of the body. The maximum permissible dose for C^{14} compounds retained in the body fats is estimated to be 250 μc, according to calculations in Handbook 52 of the National Bureau of Standards.[7] Thus, we believe we are justified in administering, over a period of several months, a maximum of five such doses to human subjects without regard to their life expectancy. It seemed unlikely that it would be necessary to exceed five doses in any study that was contemplated. For patients with limited life expectancy, we would consider it proper to administer larger amounts if necessary, but we hoped to develop methods that would permit the work to be accomplished within these conservative permissible limits.

B. *Biochemical Methods.*—Blood was drawn after administration of the tracer dose at varying times, ranging from 30 minutes to several weeks. Samples of 10 to 20 ml. obtained by venipuncture were placed in stoppered centrifuge tubes containing heparin. Each blood sample was then centrifuged; the plasma fraction was transferred as completely as possible, and its volume recorded. These fractions can then be frozen and stored until used. For shipment, each plasma sample was added to an equal volume of alcohol in a screw-cap vial; and each red cell sample was diluted with an equal volume of water and then added to twice the red cell volume of alcohol in a screw-cap vial. The samples were analyzed at either the Argonne Cancer Research Hospital or the Los Alamos Scientific Laboratory.

Plasma.—The total lipid extract was prepared by boiling the plasma sample with a total of 20 to 25 times its volume of alcohol-acetone (1:1) in three portions and filtering through a sintered glass funnel. A quantitative yield of plasma lipids was obtained. After standing at least overnight, any precipitate present in the combined total lipid extract was filtered off, and the volume was recorded.

An aliquot estimated to contain 0.5 to 1.0 mg. of free cholesterol was taken for analysis by the Sperry-Schoenheimer procedure, as modified by Sperry and Webb.[8] The remainder of the extract was treated with a slight excess of digitonin as described elsewhere,[3] and the precipitated free cholesterol digitonide was assayed for C^{14}.

The filtrates from the two free cholesterol digitonide precipitations were used, respectively, for esterified cholesterol analysis and C^{14}-assay. Each filtrate was combined with the first washing and evaporated almost to dryness on the steam bath. Then 10 ml. of alcohol and 1 ml. of KOH solution (1:2) were added, and saponification was effected by heating on the steam bath for about 1 hour. The mixture was then diluted with water to make the alcohol concentration about 50 to 70 per cent and it was extracted three times with equal volumes of petroleum ether. The combined extracts were washed thoroughly with 50 per cent alcohol. The residue from the petroleum ether extract was taken up in alcohol, and digitonin was added. In the case of the analytical samples, a large excess of digitonin was used (20 times the theoretical amount), but, for C^{14} assay, only a 25 to 50 per cent excess was used.

Red Blood Cells.—Each red cell sample was thoroughly extracted by boiling with 20 to 25 times the red cell volume of alcohol ether (3:1 mixture) in 3 portions and filtering through a sintered glass funnel. The volume of the combined extract was recorded.

Aliquots estimated to contain 0.5 mg. and 10 mg. of cholesterol were removed for cholesterol analysis and for C^{14} assay, respectively. No attempt was made to isolate the red cell esterified cholesterol fraction.

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*Shreeve[5] also reported that 56 per cent of the C^{14} in acetate was eliminated as $C^{14}O_2$ by man at the end of 24 hours. Hellman[6] reported that 60 per cent of the radiocarbon was *retained* at the end of 24 hours, and 35 per cent at the end of the first week after administration of acetate. It should be noted that he used the methyl-labeled acetate (2-C^{14}-acetate).

C. *Radioassay.*—In some experiments the cholesterol digitonide was counted as an infinitely thick sample using a thin window Geiger counter,[9] or a windowless flow counter, and in others, in a liquid scintillation counter.[10] For the latter method, the sample was dissolved in methanol, 10 ml. of toluene was added, and the mixture was evaporated to dryness on a steam bath to decompose the digitonide. The residue was then taken up in 15 ml. of toluene. One aliquot (2 ml.) was measured for cholesterol content, and a second aliquot (10 ml.) was added to 40 ml. of toluene containing 100 mg. of the scintillator. diphenyloxazole. The scintillation mixture was placed in a stoppered weighing bottle, cooled to -20° C., and counted. The efficiency of the counter was determined daily, using an external secondary standard consisting of methyl-trityl ether labeled in the methyl group. This secondary standard was calibrated against our primary standard, a solution of sodium C^{14}-carbonate supplied by the National Bureau of Standards. The primary and the secondary standards were converted to $C^{14}O_2$ and assayed in a vibrating reed electrometer. The efficiency of the scintillation counter was in the range of 56 to 60 per cent with the discriminator settings that were used. The background of the counter was approximately 130 c.p.m. Sufficient counts were taken from the sample so that the standard error of the net count per minute was less than 5 per cent. All results are expressed as specific activity in microcuries per gram.

MATERIAL

The clinical studies were conducted on patients admitted to the research wards of the Argonne Cancer Research Hospital. The subjects are described in Tables I and V. In addition to the measurements of specific activity of plasma C^{14}-cholesterol, other studies were performed on some or all of the patients, as follows: (a) excretion of $C^{14}O_2$ as a function of time after the administration of the acetate; (b) excretion of total C^{14} and of C^{14}-urea in the urine as a function of time after the dose; (c) blood level of 1-C^{14}-acetate as a function of time after the intravenous dose; (d) excretion of C^{14}-labeled corticoids in the urine; (e) specific activity of C^{14}-cholesterol in tissues removed surgically at various times after the intravenous dose.

The results of these additional studies will be described in detail in subsequent reports.

TABLE I. THE SUBJECTS WHO RECEIVED ORAL DOSES OF ACETATE

CASE NO.	UNIT NO.	SEX	AGE	DATE	DIAGNOSIS
1	57-20-31	F	37	5/ 2/53	Carcinoma of ovary
2	52-74-89	F	32	4/14/53	Malignant melanoma, posthypophysectomy
3	54-37-12	M	58	4/13/53	Carcinoma of prostate, postadrenalectomy
4	56-23-28	F	41	4/24/53	Carcinoma of breast
5a	56-44-96	M	59	2/25/53	Peripheral vascular disease
5b	56-44-96	M	59	3/ 9/53	Repeat
6a	54-35-49	M	64	4/10/53	Cor pumonale, asthma
6b	54-35-49	M	64	4/18/53	Repeat
7	58-21-56	F	39	9/15/53	Diabetes mellitus
8a	57-21-58	M	71	4/29/53	Myxedema, postthyroidectomy
9a	58-53-50	M	57	10/17/53	Myxedema, spontaneous

RESULTS

The variations in specific activity of plasma free cholesterol as a function of time after an oral dose of acetate are shown for nine patients in Table II. Although samples were obtained at times other than the intervals used in the table, only the values for 1, 2, 4, 8, 12, 24, and 48 hours are listed. When a sample was not obtained at the precise time indicated, the value was extra-

polated from an eye-fitted curve to the listed time. It is evident from plots of the data in Table II that the peak value for plasma free cholesterol occurred two to four hours after the oral dose was given. The change in the specific activity of the plasma esterified cholesterol in the same subjects are listed in Table III. Over a period of 48 hours, the specific activity of the ester fraction gradually approached the specific activity of the free fraction. Occasionally the curves crossed, but it was more usual for both to become essentially equal, and to decrease at comparable rates from about the second day on. The values for red cell free cholesterol are given in Table IV. The peak value was reached in approximately eight to twelve hours. Composite curves for the seven subjects listed in Table I are given in Fig. 1. It is evident that equilibration between the plasma free and red cell fractions took place rapidly and was usually complete by the time the red cell value reached its peak. Equilibration between these two components and the plasma ester fraction was considerably slower, usually requiring several days to reach completion. Repeat determinations were made for Cases 5 and 6, and the results of the two studies are in good agreement.

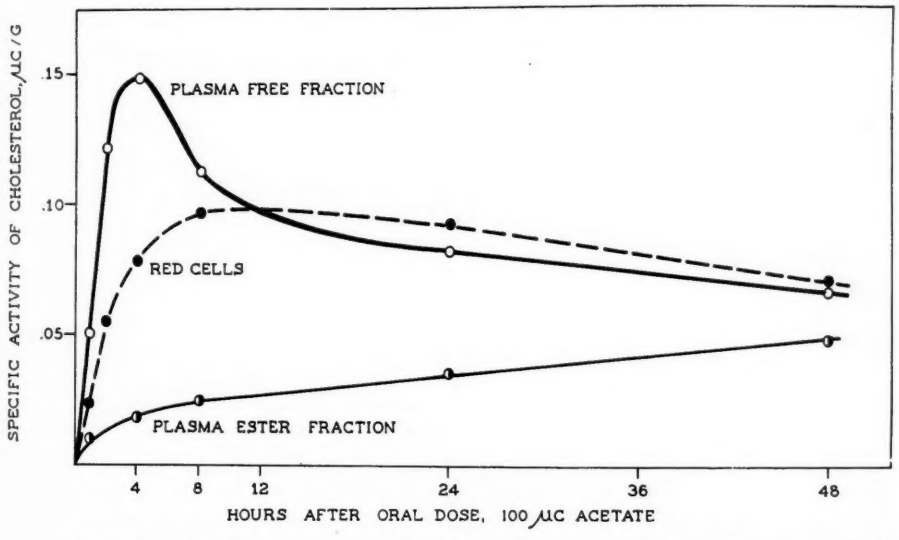


Fig. 1.—Composite curves for specific activity of cholesterol after an oral dose of 100 μc of acetate. These are the average values for the seven subjects listed in Table I. The data for the individual curves are given in Tables II, III, and IV.

Two patients with untreated myxedema were given C^{14}-acetate orally (Cases 8 and 9). Both had plasma cholesterol levels in excess of 600 mg. per cent at the time of the study. It is of interest that the rate of appearance of labeled cholesterol in blood was so slow that significant activity could not be detected with the 100 μc dose, using the Geiger counter method.

Although the free cholesterol-C^{14} released from the liver is diluted to a greater extent in hypercholesterolemic plasmas than in normal ones, this effect

TABLE II. SPECIFIC ACTIVITY OF PLASMA FREE CHOLESTEROL AS A FUNCTION OF TIME AFTER AN ORAL DOSE OF 100 μc 1-C^{14}-ACETATE

CASE NO.	SPECIFIC ACTIVITY, IN μC/GM., AT TIME, IN HOURS					
	1	2	4	8	24	48
1	0.056	0.122	0.127	0.104	0.088	0.082
2	0.098	0.207	0.212	0.172	0.109	0.090
3	0.088	0.149	0.167	0.125	0.109	—
4	0.029	0.072	0.077	0.066	0.034	0.037
5a	0.021	0.122	0.170	0.106	0.077	0.059
5b	0.064	0.151	0.218	0.162	0.109	—
6a	—	0.098	0.114	0.074	0.059	0.043
6b	0.040	0.116	0.109	0.100	0.055	0.042
7	0.017	0.050	0.150	0.120	0.096	0.092
8a	nsc*	nsc	nsc	nsc	nsc	nsc
9a	nsc	nsc	nsc	nsc	nsc	nsc
Average, excluding Cases 8a and 9a	0.051	0.121	0.149	0.114	0.082	0.064

*nsc=Not significant counts.

TABLE III. SPECIFIC ACTIVITY OF PLASMA ESTER CHOLESTEROL AS A FUNCTION OF TIME AFTER ORAL ACETATE

CASE NO.	SPECIFIC ACTIVITY, IN μC/GM., AT TIME, IN HOURS					
	1	2	4	8	24	48
1	nsc*	nsc	nsc	nsc	nsc	nsc
2	0.016	0.029	0.024	0.059	0.072	0.085
3	0.016	0.040	0.050	0.059	0.077	—
4	0.003	0.003	0.016	0.024	0.034	0.034
5a	nsc	nsc	0.011	0.024	0.043	0.045
5b	0.040	0.043	0.045	0.059	0.061	0.085
6a	nsc	0.013	0.013	0.029	0.032	0.037
6b	0.005	0.006	0.021	0.029	0.042	0.048
7	0.017	—	0.028	0.030	—	0.031
8a	nsc	nsc	nsc	—	nsc	nsc
9a	nsc	—	nsc	—	nsc	nsc
Average, excluding Cases 1, 8a and 9a	0.011	0.017	0.023	0.035	0.045	0.046

*nsc=Not significant counts.

TABLE IV. SPECIFIC ACTIVITY OF RED BLOOD CELL CHOLESTEROL AS A FUNCTION OF TIME AFTER ORAL ACETATE

CASE NO.	SPECIFIC ACTIVITY, IN μC/GM., AT TIME, IN HOURS					
	1	2	4	8	24	48
1	0.021	0.048	0.088	0.104	0.090	0.080
2	0.061	0.122	0.149	0.177	0.138	0.100
3	0.018	0.061	0.090	0.109	0.096	—
4	0.013	0.027	0.040	0.021	0.056	0.043
5a	nsc*	0.027	0.059	0.095	0.077	0.053
5b	0.034	0.053	0.066	0.100	0.143	0.120
6a	0.008	0.040	0.061	0.077	0.061	0.053
6b	0.038	0.069	0.093	0.096	0.080	0.061
7	—	—	—	—	—	—
8a	nsc	—	nsc	—	nsc	—
9a	nsc	—	nsc	—	nsc	—
Average, excluding Cases 8a and 9a	0.024	0.056	0.077	0.097	0.093	0.073

*nsc=Not significant counts.

in such myxedema patients with plasma levels about 3 times normal would be only to decrease the plasma specific activity by about 33 per cent or less.*

TABLE V. SPECIFIC ACTIVITY OF PLASMA FREE CHOLESTEROL AFTER INTRAVENOUS DOSE OF 100 μc

CASE NO.	UNIT NO.	SEX	AGE	DATE	SPECIFIC ACTIVITY, PLASMA FREE CHOLESTEROL, μc/GM. AT 4 HOURS	SPECIFIC ACTIVITY, PLASMA FREE CHOLESTEROL, μc/GM. AT 24 HOURS	DIAGNOSIS
A. Subjects With No Thyroid Disease, No Cancer, and Who Were Not Receiving Cortisone Therapy							
5c	56-44-96	M	61	12/ 9/54	0.138	0.080	See Table I, repeat
5d	56-44 96	M	61	12/19/54	0.139	0.087	Repeat
10	13-26-59	M	44	11/ 6/54	0.077	0.070	No diagnosis
11	2-59-85	F	26	3/22/54	0.146	0.091	Xanthomatosis
12	19-75-94	M	43	5/28/54	0.185	0.094	Pituitary disease?
13	44-91-29	M	23	10/ 2/54	0.105	0.069	No diagnosis
14a	50-74-42	M	28	4/26/54	0.079	0.034	No diagnosis
14b	50-74-42	M	28	12/ 6/54	0.067	0.025	Repeat
15	53-76-09	F	50	9/16/54	0.112	0.073	Rheumatoid arthritis
16	60-22-36	F	27	6/ 4/54	0.125	0.054	Scleroderma
Average					0.117	0.068	
Standard Deviation					±.034	±.022	
B. Subjects With Myxedema							
9b	58-53-50	M	57	10/28/53	0.024	0.031	See Table I, repeat
17a	51-42-92	F	52	1/26/54	0.006	0.009	Secondary, due to x-ray therapy
18a	56-51-15	F	44	3/13/54	0.038	0.009	Spontaneous
19a	55-03-36	F	49	2/ 1/54	0.012	0.010	Secondary, due to I^{131}
20a	59-78-11	F	51	4/16/54	0.037	0.025	Spontaneous
21	59-90-51	F	40	6/ 4/54	0.034	0.005	Secondary, due to I^{131}
22	58-88-12	F	41	6/ 4/54	0.013	0.022	Secondary, due to I^{131}
23a	61-32-48	F	55	11/17/54	0.020	0.007	Spontaneous
24	29-47-28	M	70	8/17/54	0.042	0.029	"Compatible with myxedema"—excluded from averages
Average					0.019	0.015	
C. Subjects Receiving Cortisone Therapy							
25a	59-81-02	F	42	5/20/54	0.498	0.266	Scleroderma
26	59-63-85	F	53	3/24/54	0.279	0.092	Scleroderma
27	56-37-84	F	44	8/25/54	0.776	0.250	Lupus erythematosus
28	47-77-21	F	29	8/25/54	0.227	0.071	Lupus erythematosus
29	51-52-54	F	47	8/ 5/54	0.897	0.114	Rheumatoid arthritis
Average					0.535	0.159	
D. Subjects With Malignancies; No Cortisone							
30	57-62-86	M	50	4/24/54	0.693	0.282	Multiple myeloma
31	47-87-97	M	67	4/24/54	0.472	0.233	Cancer of lung
32	58-46-42	F	45	8/25/54	0.492	0.205	Cancer of ovary
33	57-29-49	F	67	5/ 5/54	0.266	0.188	Cancer of ovary
34	28-16-87	F	64	2/22/54	0.310	0.103	Hypernephroma
Average					0.447	0.202	

*Liver free and esterified and red cell cholesterol concentrations are reported to be essentially normal in hypothyroidism; equilibration between free cholesterol in liver and plasma appears to proceed at a normal rate, as judged by animal studies.[13] Consequently the cholesterol-C^{14} molecules are equally distributed first, between the liver-plasma free cholesterol mass, estimated as 6.05 Gm. in the normal and 9.55 Gm. in patients with a threefold elevation of the plasma level, and a little later among the liver, plasma, and red cell fractions amounting to 9.05 and 12.55 Gm. in the normal and the hypercholesterolemic case, respectively.

This is strong evidence that the rate of synthesis in liver was very slow. The effects of various therapeutic measures on the rate of cholesterol synthesis in myxedema will be described in a subsequent paper.

Inspection of our data seemed to indicate that adequate information on the apparent rate of synthesis could be obtained from determination of the specific activity of plasma free cholesterol 4 hours and 24 hours after the tracer dose. Since the rate of absorption of an oral dose could modify the results, the effect of the route of administration of acetate was investigated.

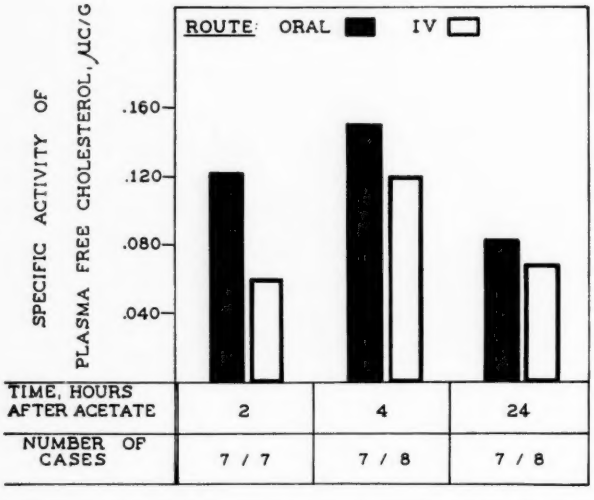


Fig. 2.—Influence of route of administration of radioactive acetate on specific activity of plasma free cholesterol-C^{14}.

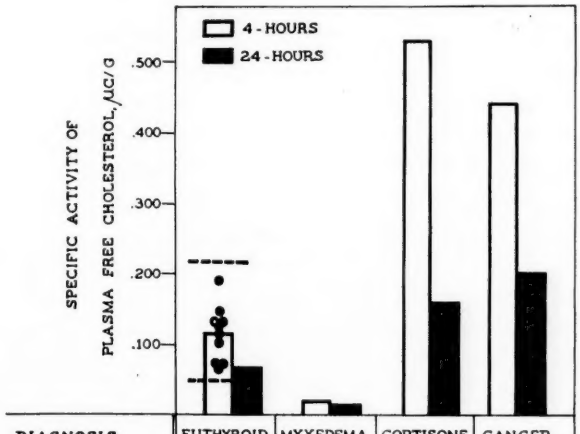
After intravenous administration of 1-C^{14}-acetate, the specific activity of plasma free cholesterol was determined 4 and 24 hours later. The results are shown in Table V, *A* for a group of subjects who had neither thyroid disease nor cancer, and who were not receiving therapeutic doses of cortisone at the time of the test. The average values at 4 and 24 hours for this group are approximately 80 per cent of the values observed in the first group (Tables I and II), who received the acetate orally. In Case 5 the average 4-hour value for two oral doses was 0.190 μc per gram, and for two intravenous doses given 20 months later, it was 0.139 μc per gram. The ratio of intravenous to oral in this case was 0.74. By either route of administration the ratio between the average 24-hour value and the average 4-hour value is substantially the same: 0.55 and 0.58 for oral and intravenous, respectively. This suggests that the route of administration does not affect the distribution of the labeled cholesterol. The higher values observed when the dose is given orally is readily explained by the fact that more intact acetate can reach the liver promptly via the portal blood than is the case when the material is given intravenously. The average values for the plasma free fraction after oral and intravenous

administration of the tracer are shown in the block diagrams of Fig. 2. Evidence from comparable experiments in dogs[3] shows that the time of the peak value is not affected by the route of administration. At the present time all our routine studies are performed using the intravenous dose of acetate.

The rate of decrease in specific activity subsequent to equilibration was much slower than the original rate of increase. In some patients the subsequent decline was followed for as long as 19 days, but the data were not suitable for satisfactory mathematical treatment.

As we accumulated experience with the procedure it was evident that the peak value (the 4-hour value) varied greatly, depending upon the metabolic status of the subjects. It is possible to discern several patterns of response that are of interest:

1. The euthyroid individual without obvious metabolic abnormality, and who was not receiving therapeutic doses of cortisone. The findings for this group are given in Table V, *A*, and have been discussed above. After an intravenous dose of 100 μc of acetate, the peak value for specific activity of plasma free cholesterol is approximately 0.120 μc per gram. It is our impression that values within the "normal" range probably do not differ from the average by more than a factor of 2, i.e., from 0.06 to 0.240 μc per gram.



DIAGNOSIS	EUTHYROID	MYXEDEMA	CORTISONE	CANCER
NUMBER OF CASES	8	9	5	5
AVERAGE VALUE FOR 4 HR. SAMPLE, μC/G	0.117	0.019	0.535	0.447

Fig. 3.—Specific activity of plasma free cholesterol-C^{14} after intravenous acetate in patients with different patterns of response. For the euthyroid subjects, the individual values are shown by the solid circles. Variations from the average by a factor of 2 are shown by the dotted lines.

2. The patient with frank clinical myxedema, whether spontaneous or induced. The data for nine such patients are given in Table V, *B*. The average value for the 4-hour peak is 0.019 μc per gram, and for the 24-hour period, 0.015 μc per gram. It is of interest that the ratio of the 24-hour to the 4-hour

level is 0.80, as compared with a ratio of 0.58 in the case of the euthyroid individuals. In the group with myxedema the average value for the 4-hour peak is about one-sixth of the average for the euthyroid subjects.

3. Patients treated with cortisone. In Table V, *C* are given the data for five patients with collagen diseases who were under treatment with substantial doses of cortisone. The average 4-hour value for these patients was 0.535 μc per gram, or nearly 5 times that of the euthyroid group (Table V, *A*). The ratio of the 24-hour level to the 4-hour for the cortisone group was 0.30 as compared to 0.58 for the former group.

4. Certain patients with widespread malignancies. Among a group of 16 patients with cancer in various stages who were studied, there were five with abnormally high values for cholesterol synthesis. These data are given in Table V, *D*. The average value for the 4-hour level in this group was 0.447 μc per gram, and for the 24-hour period, 0.202 μc per gram.

The results of all the intravenous studies reported are compared graphically in Fig. 3. Detailed discussion of these variations, and speculations as to the reasons for them, are beyond the scope of this paper, but will be considered in later reports. It is sufficient to note here that remarkable variations have been observed which merit further consideration.

DISCUSSION

The results demonstrate the feasibility of carrying out repeated studies of cholesterol synthesis in human subjects using doses of acetate as small as 100 μc. The specific activity of cholesterol in the various blood fractions is sufficiently high following oral or intravenous administration of this amount of radioactivity to make it possible to obtain reasonable counting accuracy with either a Geiger counter or a liquid scintillation counter. When the ratio between background and net counts is not great, as with these samples, the time of count must be long to yield significant data. The difficulty can be overcome in two ways: (1) larger samples of cholesterol can be counted, which require the drawing of larger samples of blood, or (2) larger doses of acetate must be used. Increasing the dose to 200 μc improves the counting accuracy considerably, and unless a series of doses is to be given in rapid succession, it does not exceed the permissible dose retained in the body. For short-term studies, where attention is directed only to the first day or so after administration of the acetate, the dose of 100 μc is adequate. If, however, it is desired to follow the plasma cholesterol-C^{14} for several weeks to measure turnover accurately, the larger dose, 200 μc, should be used.

Comparison of these results with those from dogs reported elsewhere[2, 3] shows a great similarity in pattern. The principal difference is that the peak values were reached more slowly in man. The plasma free value reached a peak in about 1 hour in dogs and in 2 to 4 hours in man; the red blood cell values at about 4 to 6 hours in dogs, and 8 to 12 hours in man.

By taking serial liver biopsies in dogs at various times after the injection of C^{14}-acetate, the rates of appearance of cholesterol-C^{14} in both the liver free and plasma free fractions were followed.[3] It was found that hepatic choles-

terol synthesis was completed in less than an hour from small doses of injected C^{14}-acetate. Beeckmanns and de Elliott have reported peak values in 30 minutes in mice.[14] Similar studies in hepatectomized dogs showed that the cholesterol-C^{14} appearing in the plasma free fraction was derived from the liver.

The rapidity with which the peak plasma specific activity values were reached cannot be understood on the basis of the reported "turnover" times of about 9 to 12 days for both plasma and liver cholesterol. There must be a very rapid molecular interchange or mixing of free cholesterol molecules between liver and plasma, but with no change in level. The half-time for this equilibration process in the dog was estimated to be about 20 minutes. In man it would be reasonable to assume a half-time of about 1 hour as a maximum value, since the plasma specific activity curve reaches its peak about three times as slowly in man as in the dog.

The normal rate of movement of free cholesterol molecules between liver and plasma may be estimated from these data. Taking the amount of free cholesterol in liver as 4.3 Gm. and in plasma as 1.75 Gm., it is apparent that after equilibration has been reached, the amount of labeled cholesterol in liver is 71 per cent of the total amount formed in the liver ($4.3 \times 100/4.3 + 1.75$), neglecting the small amounts of labeled cholesterol that have left the liver for fractions other than the plasma free during this initial period. Thus, 29 per cent of the labeled cholesterol in liver must have entered the plasma and since there is at present no evidence indicating that the kinetics of newly synthesized free cholesterol molecules are different from "older" ones, it is reasonable to assume that 29 per cent of 4.3 Gm., or 1.25 Gm., has moved across in a time given by the equilibration time, or 1.44 hours. From this it follows that at least 0.87 Gm. of free cholesterol must be moving across the liver-plasma boundary each hour in each direction.

If the delay in the peak plasma value is due to a slower completion of synthesis of cholesterol-C^{14}, the equilibration rate would be more nearly as rapid as in the dog, and the estimate of 0.87 Gm. would be considerably increased, so this must be taken as a minimum value. This lability of individual molecules may play a role in the regulation of the plasma cholesterol level. It has been reported that the rate of cholesterol synthesis in liver is strongly influenced by the concentration of cholesterol in liver.[9] The rapid rate of movement of molecules between liver and plasma provides a mechanism whereby changes in plasma concentration may be reflected by changes in liver concentration and result in changes in the rate of hepatic cholesterol synthesis.

The specific activity-time curve that is obtained after administration of C^{14}-labeled acetate appears to have value as a clinical laboratory test. The ascending portion of the curve (i.e., the first 3 to 4 hours) can be taken for normocholesterolemic plasmas as a *relative* expression of the rate of cholesterol synthesis in the liver, the rate of movement of labeled molecules from liver into the plasma free fraction, and the rates of movement from this fraction into red cells and various extrahepatic tissues.[13] It is probably correct

to use the peak value as an integrated expression of these functions. For practical purposes, the clinically important variable, whose magnitude is suggested, is the rate of cholesterol synthesis in the liver.

The next portion of the curve, that is, the period from 4 hours to about 48 hours, represents the equilibration of the free cholesterol in plasma, red cells, and liver with that in extrahepatic tissues, especially the visceral organs, and also with the plasma esterified cholesterol, as has been shown in animal studies.[15] Some conversion into bile acids and excretion also occurs during this period. This portion of the curve is probably not clinically useful.

When labeled acetate is used as a precursor of cholesterol, it does not seem possible to derive data which will permit an estimate of the size of the rapidly exchangeable pool of cholesterol in the body. Such an estimate should be possible after the intravenous administration of labeled cholesterol, and values for turnover have been determined in this way by Kurland, Lucas, and Freedburg,[12] and in our own laboratory. The number of cases where pool size was measured with cholesterol, and the synthesis rate with acetate are too few and the results are too varied to permit drawing any conclusions at the present time.

The fraction of the dose of acetate utilized for cholesterol synthesis by the liver has been estimated for each time interval and the average value for Cases 1 through 6 are given in Table VI. The maximum value, 1.4 per cent, must be considered the most nearly accurate since labeled cholesterol moves continuously out of the liver-blood system into other tissues, but not in the other direction. The fraction of acetate utilized for cholesterol synthesis in

TABLE VI. PER CENT OF DOSE OF C^{14}-ACETATE PRESENT IN TOTAL LIVER AND BLOOD CHOLESTEROL AT VARIOUS TIMES

CASE NO.	TIME AFTER DOSE IN HOURS					
	1	2	4	8	24	48
1	—	—	—	—	—	1.08
2	1.29	2.24	1.90	1.85	1.39	1.22
3	1.05	1.60	1.63	1.34	1.28	—
4	0.36	0.71	0.67	0.58	0.51	0.50
5a	—	—	1.32	1.06	0.89	0.68
5b	0.926	1.60	1.77	1.56	1.36	1.35
6a	—	1.00	0.97	0.81	0.68	0.58
6b	0.56	1.22	1.06	1.04	0.75	0.68
Average*	0.84	1.40	1.33	1.20	1.05	0.87
Ratio of $\frac{\text{total } \mu c}{\text{plasma-free } \mu c}$	7.5	6.0	5.1	5.9	7.6	
Ratio of $\frac{\text{total } \mu c}{\text{plasma-free S.A. in } \mu c/\text{Gm.}}$			8.9		13.2	

*Excludes Case 5a.

Calculations are based on the following estimates and assumptions:

(1) That the total amounts of cholesterol in the various fractions is the same in all patients and is, liver free 4.3 Gm., liver esterified 0.7, plasma free 1.75, plasma esterified 3.50, and red blood cell free 3.0.

(2) That the liver free cholesterol specific activity is 2 times that of plasma free at 1 hour, 1.5 times at 2 hours, and is equal at all later times.

(3) That the liver esterified specific activity is 0.5 that of plasma free at 1 hour, 0.5 at 2 hours, 0.6 at 4 hours, 0.6 at 8 hours, 0.8 at 24 hours, and equal at 48 hours. These equilibration values are based on experimental values for dogs and the assumption that the liver-plasma equilibration rates are 2 to 3 times as slow in human beings as in dogs.

extrahepatic tissues is undoubtedly larger than the values for the liver-blood system since in rats it was found to be 3 times as large,[2] but cannot be estimated from the data available for man.

It is apparent from Table VI that the total amount of labeled cholesterol in the liver-blood system decreases rather slowly from the peak value at 2 hours, reaching a decay half-time between 24 and 48 hours of over 3 days. Extrapolation to zero time indicates that not more than 1.5 per cent of the acetate-C^{14} was utilized for hepatic cholesterol synthesis.

The total per cent of C^{14}-acetate converted into cholesterol in the liver may be estimated for the several groups of subjects in Table V by analogy with the data presented in Table VI. Using the assumptions given in the footnote to this table, the ratio between the total activity of cholesterol of liver and blood (expressed in microcuries) and the specific activity of plasma free cholesterol (expressed in microcuries per gram) was found to be 8.9 at 4 hours and 13.2 at 24 hours. These ratios, multiplied by appropriate values for specific activity of plasma free cholesterol at 4 and 24 hours, respectively, can be used to estimate the per cent of the tracer dose converted into hepatic cholesterol. For the 7 normal individuals (Table V, *A*) who were euthyroid, free of cancer, and who were not taking cortisone, the result was 1.04 per cent at 4 hours, and 0.90 per cent at 24 hours; for the myxedema patients (Table V, *B*), 0.23 at 4 hours and 0.26 at 24 (corrected for hypercholesterolemia); for the cortisone-treated patients (Table V, *C*), 4.75 at 4 and 2.10 at 24 hours; and for the cancer patients (Table V, *D*) 3.98 at 4 hours and 2.66 at 24 hours.

Hellman, Rosenfeld, and Gallagher[4] estimated that a minimum of 3 per cent of the administered 2-C^{14}-acetate was incorporated into the liver-blood cholesterol. They studied only cancer patients and concluded that this disease does not alter cholesterol metabolism. Our figures for cancer patients agree well with their values, but our figures for normals are definitely lower. Our results indicate a decrease in hepatic cholesterol synthesis in myxedema from 1.0 per cent to 0.23, an increase in cancer to 4 per cent, and an increase following cortisone therapy to 4.75 per cent.

SUMMARY AND CONCLUSIONS

1. The hepatic synthesis and kinetics of cholesterol have been studied following the oral or intravenous administration of 1-C^{14}-acetate one or more times in 34 patients.

2. In man, the peak of the specific activity-time curve for plasma free cholesterol occurs 2 to 4 hours after a single dose of acetate by either route.

3. The rate of hepatic cholesterol synthesis can be roughly estimated from the maximum plasma free cholesterol specific activity value for normocholesterolemic patients. A correction is necessary for hypo- or hypercholesterolemic patients. The per cent of the dose of C^{14}-acetate incorporated into the total liver-blood cholesterol may be estimated and is also an index of hepatic cholesterol synthesis.

4. The rate of interchange of free cholesterol between liver and plasma has been estimated to be at least 0.87 Gm. per hour in each direction.

5. In man, the cholesterol-C^{14} in the red cells comes into equilibrium with plasma free cholesterol-C^{14} in about 8 to 12 hours. The plasma esterified cholesterol is derived from the free fraction at a slower rate, so that equilibrium between the two is seldom completed before 24 to 48 hours after the administration of the tracer dose.

6. The specific activity of plasma cholesterol may be followed for several weeks after a single oral or intravenous dose of 100 to 200 μc of acetate.

7. The fraction of the dose of carboxyl-labeled acetate that is utilized for cholesterol synthesis was found to be approximately 1.04 per cent for normal human beings, 0.23 for myxedema patients, 4.0 for cancer patients, and 4.75 for cortisone-treated patients. It is apparent that the metabolic state of the subject produces marked variations in the rate of hepatic cholesterol synthesis.

8. Carbon-14-labeled acetate can be used to study cholesterol metabolism in man, using doses that do not expose the subject to hazardous amounts of radiation.

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BLOOD LEVELS OF 17-HYDROXYCORTICOSTEROIDS IN NORMAL AND TOXEMIC PREGNANCIES

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With the Technical Assistance of Margaret Henley

SELYE[1] has proposed the hypothesis that pre-eclampsia and eclampsia may be the result of continued hyperfunction of the adrenal cortex caused by stress. This hypothesis has received support from various investigators.[2-4] Venning,[5] using a bio-assay, found increased excretion of glucocorticoids during the last trimester of normal pregnancy. In toxemia of pregnancy, Tobian[6] found the excretion of formaldehydogenic corticoids to be higher than that of normal pregnancy. Chart, Shipley, and Gordon[7] noted no significant changes in the excretion of formaldehydogenic steroids in toxemia, but found a steroidal fraction with a marked sodium-retaining activity. Lloyd and his co-workers[8] observed a considerable increase in both the "freely water-soluble fraction" and the "poorly water-soluble fraction" in pre-eclampsia and eclampsia. They suggested that the former fraction might consist of cortisone and 17-hydroxycorticosterone and the latter of desoxycorticosterone. Venning[9] has recently reported that the glucocorticoid excretion in toxemia was lower than that of normal pregnancy, but that the excretion of formaldehydogenic steroids and particularly the mineralocorticoids was higher. All of these studies on urinary adrenal cortical steroids would appear to indicate an elevated rate of excretion in toxemia of pregancy as compared with that of normal pregnancy. Since some of these data are for the excretion of mineralocorticoids only, and others for the excretion of formaldehydogenic steroids which include the mineralocorticoids as well as the glucocorticoids, it may well be that it is an increase in the mineralocorticoids which is being measured by all observers. However, since the methods for determining urinary corticoids are not reliable from the quantitative viewpoint,[10, 11] the significance of these increased values might be questioned.

Recently, Nelson and Samuels[12] devised an accurate quantitative method for determining the *blood* corticoids which give the Porter-Silber reaction. Using this method, Gemzell[13] found that the blood levels of 17-hydroxycorticosteroids in normal pregnancy are much higher than those of normal nonpregnant women and that these levels rise progressively until the end of gestation.

The purpose of the present study was to collect data for the blood levels of 17-hydroxycorticosteroids in normally pregnant patients and in patients with

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toxemia of pregnancy. It was thought that this study might aid in determining what types of adrenal cortical steroids, if any, are concerned with toxemia of pregnancy. The effect of labor on the levels of the 17-hydroxycorticosteroids was also observed.

MATERIALS AND METHODS

The studies were performed on 69 patients distributed as follows: 32 normal pregnant patients not in labor, 8 normal pregnant patients in labor, 19 patients with pre-eclampsia, 4 with prepartum convulsive eclampsia, 2 with postpartum convulsive eclampsia, and 4 with essential hypertension associated with pregnancy. Pertinent data on the age and length of gestation are given in Tables I, II, and III. All of the patients were in the hospital at bed rest prior to and during the collection of blood samples. Since diurnal variations in the levels of 17-hydroxycorticosteroids have been observed,[14] an effort was made to collect the blood specimens at the same time each day, namely, between 11:00 A.M. and 1:00 P.M. While this was possible with the normally pregnant patients, it was difficult to follow in the patients with toxemia of pregnancy from whom blood was withdrawn at any time during the acute phase of the disease.

The method selected for the determination of the plasma 17-hydroxycorticosteroids was a modification of the Nelson and Samuels technique devised by Bayliss and Steinbeck.[15] The correction for interfering color employed in this method seemed more justifiable than did the one used in the Nelson and Samuels procedure. However, shortly after adopting the procedure of Bayliss and Steinbeck we concluded that it was more desirable to run the assays in *duplicate* than it was to include the correction for the sulfuric acid reagent (the concentration of 17-hydroxycorticosteroids in blood is not great enough to permit both to be done, except during the latter part of pregnancy). The percentage of error introduced by eliminating this correction appeared to be less than the possible error which might arise from the assay of a single sample. We introduced a second variation in the method by substituting 95 per cent ethanol for the methanol used in the assay. This was done as a result of the finding of LaBrosse[16] that 95 per cent ethanol gives more consistent results and higher optical density than does methanol.

RESULTS

Table I presents data on the patients with normal pregnancy who were not in labor. In this group, the plasma levels of 17-hydroxycorticosteroids varied from 26.8 to 84 μg per 100 ml. of plasma with an average of 49. Table II lists data on the normally pregnant patients who were in labor. The plasma levels in this group varied from 59 to 111 μg per 100 ml. of plasma with an average of 80.9. The lower values were obtained from patients who were in the beginning of the first stage of labor whereas the higher levels were from patients in the second stage. Table III shows data on the patients with pre-eclampsia, eclampsia, and essential hypertension associated with pregnancy. Altogether, the plasma level of 17-hydroxycorticosteroids were not different from those of normal pregnancy, varying from 29.7 to 76.7 μg per 100 ml. of plasma with an average of 49.9. One patient with eclampsia (Case 25) had a plasma level of 112.3 μg which was the highest observed in the whole series, but this patient was in frank labor when the blood was obtained, and this high figure was not significantly different from those of normally pregnant patients in labor. Following delivery, and while she still had all of the symptoms of toxemia except the convulsions, her plasma level fell to 35 μg.

In Fig. 1 are shown the plasma levels of 17-hydroxycorticosteroids in normal and abnormal pregnancies plotted against the length of gestation. Although

TABLE I. NORMAL PREGNANCY

PATIENT NO.	AGE	LENGTH OF GESTATION IN WEEKS	PLASMA LEVEL MICROGRAM/100 ML. PLASMA
1.	28	30	35.5
2.	32	40	26.8
3.	24	32	38.6
4.	29	28	44.8
5.	24	34	46.5
6.	25	32	37.8
7.	35	40	51.2
8.	20	40	47.2
9.	24	38	37.6
10.	31	36	51.3
11.	32	40	51.5
12.	24	28	38.3
13.	27	38	66.1
14.	18	37	54.7
15.	24	40	54.0
16.	30	39	60.5
17.	28	33	46.9
18.	20	32	63.0
19.	28	33	52.6
20.	31	36	61.5
21.	22	37	51.6
22.	23	35	48.7
23.	33	38	63.4
24.	29	35	33.6
25.	23	36	54.7
26.	32	40	30.0
27.	42	36	64.8
28.	36	38	84.3
29.	25	40	41.4
30.	34	40	49.0
31.	38	36	33.6
32.	36	38	46.5
AVERAGE			49.0

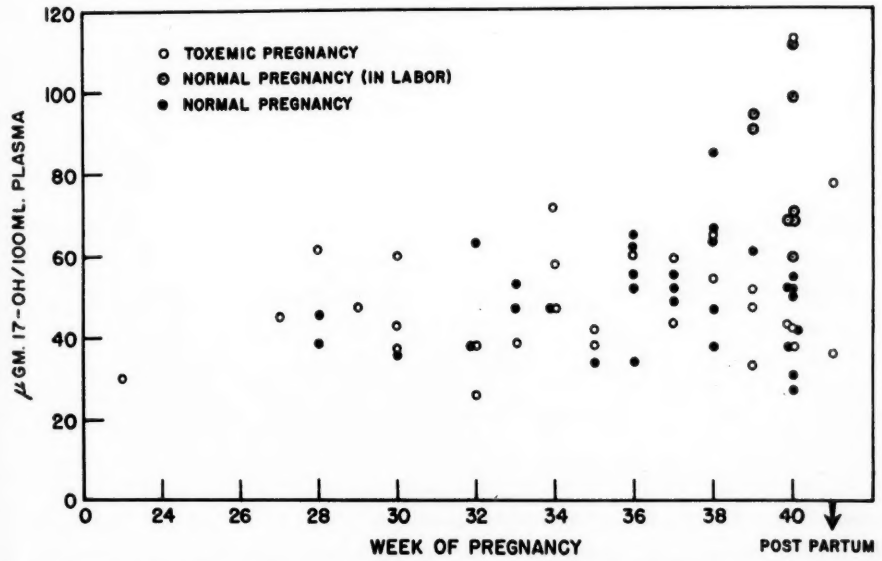

Fig. 1.—Blood levels of 17-hydroxycorticosteroids in normal pregnancy, toxemia of pregnancy, and essential hypertension associated with pregnancy, plotted against length of gestation.

TABLE II. NORMAL PREGNANCY IN LABOR

PATIENT NO.	AGE	LENGTH OF GESTATION IN WEEKS	PLASMA LEVEL MICROGRAM/100 ML. PLASMA
1.	32	40	97.4
2.	24	40	67.8
3.	29	39	90.3
4.	16	40	67.7
5.	17	40	59.3
6.	20	39	83.7
7.	18	40	111.0
8.	26	40	70.0
AVERAGE			80.9

TABLE III. PATIENTS WITH TOXEMIA OF PREGNANCY

PATIENT NO.	AGE	LENGTH OF GESTATION IN WEEKS	PLASMA LEVEL MICROGRAM/100 ML. PLASMA	DIAGNOSIS
1.	33	23	29.7	Essential hypertension
2.	32	34	46.8	Essential hypertension
3.	33	37	43.1	Essential hypertension
4.	32	40	41.8	Essential hypertension
5.	22	30	36.8	Pre-eclampsia
6.	28	30	69.9	Pre-eclampsia
7.	27	38	53.6	Pre-eclampsia
8.	29	38	64.5	Pre-eclampsia
9.	32	32	38.1	Pre-eclampsia
10.	18	32	36.0	Pre-eclampsia
11.	37	40	37.4	Pre-eclampsia
12.	25	39	51.4	Pre-eclampsia
13.	27	34	71.2	Pre-eclampsia
14.	25	36	60.2	Pre-eclampsia
15.	18	40	42.0	Pre-eclampsia
16.	30	39	32.9	Pre-eclampsia
17.	25	27	45.2	Pre-eclampsia
18.	24	39	46.8	Pre-eclampsia
19.	29	34	55.0	Pre-eclampsia
20.	33	36	37.6	Pre-eclampsia
21.	34	32	38.8	Pre-eclampsia
22.	42	34	57.6	Pre-eclampsia
23.	27	37	37.2	Pre-eclampsia
24.	17	28	61.5	Eclampsia
25.	17	40	112.3	Eclampsia
26.	24	29	46.7	Eclampsia
27.	17	30	42.6	Eclampsia
28.	17	—	35.8	Eclampsia post partum
29.	30	—	76.7	Eclampsia post partum
AVERAGE			49.9	

the data are somewhat scattered, it is clear that for any given length of gestation, the values for normal pregnancy and toxemia of pregnancy are of the same magnitude.

DISCUSSION

The present data corroborate Gemzell's[13] findings that the blood levels of 17-hydroxycorticosteroids in *normal* pregnancy during the last trimester of gestation are considerably elevated. Our data also show that the levels of these glucocorticoids in *toxemia* of pregnancy, including the severest form of

convulsive eclampsia, are similar to those of normal pregnancy. This fact would suggest that if any abnormal functioning of the adrenal cortex exists in toxemia, it does not seem to involve the secretion of glucocorticoids. When one compares the data for toxemia of pregnancy obtained in our study with the data of other investigators,[6-9] one must bear in mind the difference in the body fluids analyzed and the degree of specificity of the assays used. Chart and associates,[7] Lloyd and co-workers,[8] and Venning[9] all found an increase in the mineralocorticoids which would suggest that the increase in formaldehydogenic steroids found by Tobian[6] might also be due to an increase in the mineralocorticoid fraction. By excluding the glucocorticoids from involvement in toxemia, our results lend support to the work of the afore-mentioned investigators which indicates that mineralocorticoids may be the hormones involved in this abnormality.

The sources of the elevated levels of 17-hydroxycorticosteroids in pregnancy deserve some speculation. High levels of these hormones have been observed in patients under the stress of surgery[17] and also in patients with liver disease.[18] It has been suggested that the determinants of the blood levels of adrenal hormones are the rate of their production by the adrenal glands and the rate of their metabolism by the tissues, particularly the liver. Gemzell[13] has postulated that the increased production of 17-hydroxycorticosteroids in pregnancy may be due to adrenal stimulation in the mother and fetus by the high levels of estrogens in the maternal blood. If the adrenal glands are the only source of the increased plasma levels of 17-hydroxycorticosteroids in pregnancy, it might be assumed that maximal stimulation of these glands by endogenous factors has occurred since some of the levels observed are comparable to those seen in normal nonpregnant individuals during maximal stimulation by exogenous ACTH. That this may *not* be the case is evident from the fact that a further rise in the blood levels of 17-hydroxycorticosteroids was observed during labor. Furthermore, studies now in progress seem to indicate that stimulation of the adrenal cortex by the intravenous administration of ACTH to a pregnant woman evokes a further rise in the plasma levels of 17-hydroxycorticosteroids similar to that produced in normal nonpregnant subjects.

All these facts speak against maximal stimulation of the adrenal cortex during pregnancy and leave two alternatives to explain the high levels of 17-hydroxycorticosteroids: first, that the metabolism and tissue uptake of these hormones is impaired; and second, that sources other than the adrenal glands might be producing these hormones during pregnancy. Although there is no special reason to consider the first possibility seriously, it will be investigated in our laboratories. The second possibility is favored by the evidence currently available. It has been shown recently that the placenta produces not only estrogen and progesterone, but also adrenocorticotropic and cortisone-like substances.[19, 20] These hormones are probably necessary for a successful outcome of the reproductive process and their secretion by the placenta might provide a certain degree of protection against eventual alteration in their output by the maternal endocrine glands. Thus, it is possible that the elevated blood levels of 17-hydroxycorticosteroids in pregnancy are due, at least in part, to a production of these hormones by the placental tissues.

SUMMARY

1. Blood levels of 17-hydroxycorticosteroids (glucocorticoids) were studied in normal pregnancy, toxemia of pregnancy, and essential hypertension associated with pregnancy.

2. In all three groups the levels of these corticoids were found to be elevated over those of normal nonpregnant women with no difference being noted among the various groups.

3. Labor was found to produce an even greater rise in the levels of these corticoids.

4. The possibility that the placenta may be concerned with the production of the high levels of these corticoids during pregnancy is discussed.

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THE ANTIMALARIAL ACTION OF PRIMAQUINE AGAINST THE BLOOD AND TISSUE STAGES OF FALCIPARUM MALARIA (PANAMA, P-F-6 STRAIN)

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THIS study was designed to make a semiquantitative evaluation of the antiparasite spectrum of primaquine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline, against several stages of *Plasmodium falciparum.* In addition to the scientific interest of such information, certain questions have developed as a result of the increased use of primaquine as a curative and prophylactic agent in vivax malaria and the possibility that primaquine will be used in the field against *Plasmodium falciparum* coincidental to its use in vivax infections.

General Procedures.—The test strain of falciparum malaria used was isolated originally in a mestiza from El Limon, Panama, and has been designated by Jeffery and co-workers[1] as P-F-6. This strain has had intermittent exposure to chloroquine (Aralen) and chlorguanide (Paludrine) and has demonstrated a capacity for very rapid development of resistance to pyrimethamine (Daraprim).[2] Primaquine was given orally as the diphosphate salt with doses expressed in terms of base. The infections were produced by mosquito bites on nonimmune white volunteers at Stateville Penitentiary, Joliet, Illinois. The penitentiary is in a nonendemic area. The general method of infection and follow-up have been reported previously by Alving and co-workers.[3]

EFFECT OF PRIMAQUINE ON THE TISSUE STAGES OF FALCIPARUM MALARIA

I. *Continuous Therapy in the Incubation Period (1-1-6).*—Our preliminary experiment on the action of primaquine against tissue stages was performed by the classical method of testing for causal prophylaxis as originally devised by James.[4] In this method drug is given on the day before inoculation, on the day

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of inoculation, and on each of the next six days. Five nonimmune volunteers were treated by 30 mg. primaquine daily on this schedule and all five were protected after being bitten by infected mosquitoes.

A group of nine men was also bitten by infected mosquitoes in rotation with adequate controls and were treated by 30 mg. primaquine semiweekly for 4 weeks. All nine men were protected against the infection. Drug was given on day 0 and day 4 of each week.

II. *Single Dose Therapy.*—

A. *Theory:* The unexpected effectiveness of primaquine against the tissue stages of *Plasmodium falciparum* by the orthodox testing program mentioned previously suggested the possibility of studying the changes occurring in the incubation period in terms of the susceptibility of the parasite to primaquine. It would seem likely that the early tissue stages undergo radical changes in their metabolism preparatory to developing circulating blood forms, and consequently they would vary in response to the drug on different days during the prepatent period.

B. *Duration of prepatent period:* In order to explore this possibility it was first necessary to establish the period in which only tissue forms exist. From the subinoculation experiments of Fairley[6] it appears likely that blood forms of *Plasmodium falciparum* malaria in the Southwestern Pacific do not develop before 144 hours post inoculation. The experiments of Jeffery and associates,[7] using the same strain as we have employed in the present study, bear on this same point. In Jeffery's experiments volunteers were infected by the equivalent of 2,000 mosquito bites, and daily blood smears were made. Even after such massive inoculations, trophozoites were not detectable until day 7.*

To be more certain of the exact interval in which trophozoites are not present, we tested 10 men after a standard inoculation of sporozoites by withdrawing 100 to 500 c.c. of blood for immediate subinoculation to 10 other men of homologous blood groups and types. Blood was drawn on day 5 (122 hours post inoculation) from 5 men and on day 6 (144 hours post inoculation) from 5 men. In no case did these large subinoculations transmit the disease to the recipient of the blood. We concluded from this observation that under the conditions of our experiments the parasites do not finish their metamorphosis in the tissue until after day 6 (144 hours post inoculation).

C. *Duration of drug action:* It is apparent then that any attempt to measure the action of a drug against tissue forms of *Plasmodium falciparum* must be limited to the short period of six full days after inoculation of sporozoites. Our present knowledge of the pharmacology of primaquine suggests that this drug rapidly disappears from the blood. Blood levels of a heptane soluble diazotizable fraction determined after the administration of 30 mg. primaquine show an initial rise to a maximum value in about six hours with a very rapid fall to undetectable levels in 24 hours.[2] Although evidence is incomplete it

*Although an estimated 20,000 bites were reported in this study, personal communication with the authors indicate that only 10 to 15 per cent were actually administered on the first day.

would appear that a measure of the susceptibility of the tissue forms to primaquine could be made for short periods, probably for each 24-hour period, during the six days of the incubation period.

D. *Procedures:* Three separate days of the incubation period were selected for an evaluation of the effect of one dose of primaquine administered on a single day. The first, third, and fifth postinoculation days were arbitrarily chosen for comparison. The relative effect of four different doses was studied: 10, 15, 30, and 45 mg. of base. For these investigations 30 groups of four men and five groups of two were infected. The volunteers in each group were bitten in rotation by ten infected mosquitoes. An interrupted biting technique was employed.[3] One man from each group served as a control; his "bite-partners" were treated by one of the four doses of drug tested, each man, however, receiving his single dose of drug on a different day (1, 3, or 5 post inoculation).

E. *Results and interpretations:* The information obtained from this series of experiments has enabled us to plot the "drug-dosage response curve" for primaquine given on a single day (Fig. 1) as well as the "parasite-age response curve" for a given dose of primaquine (Fig. 2). Therapy on day 3 appears to have the greatest effect for doses less than 30 mg. primaquine base. In the case of the 30 mg. dose the difference in the percentage protected between day 3 and day 1 is probably not significant; treatment on both days was highly effective (causal prophylaxis). In marked contrast to day 1 and day 3, all dosages up to 45 mg. were ineffective if administered on day 5.

Fig. 1 illustrates the fact that on each of the three test days the response to a graded series of doses gives a differently shaped curve. The curves in both Fig. 1 and 2 support the interpretation that a series of changes in susceptibility of the tissue forms of *Plasmodium falciparum* to primaquine is occurring throughout the incubation period.

EFFECT OF PRIMAQUINE ON THE CIRCULATING BLOOD FORMS OF FALCIPARUM MALARIA

I. *Asexual Stages.*—Inasmuch as primaquine has a relatively weak action against the trophozoites of vivax malaria, it was not expected to have a remarkable action against the trophozoites of falciparum malaria. To determine the extent of this action a group of six nonimmune white men were inoculated by bites of mosquitoes infected with the test strain of falciparum malaria. These men were observed closely by thick blood smear examinations and were left untreated until blood forms were demonstrated on two successive days by the thick smear method of Earle and Perez,[8] at which time treatment with primaquine was instituted. Parasites are detectable with this technique when they appear in a concentration of about 10 per cubic millimeter.

Four of the six men used to study the effect of primaquine on asexual blood forms (trophozoites) of falciparum were given 30 mg. of primaquine base daily (Fig. 3). In three of these four men primaquine was grossly inadequate for control of the parasitemia and the clinical symptoms. It was felt that all three became too ill to justify continuation of the experiment and their infection was accordingly terminated by chloroquine. Variations in the clinical course of falciparum malaria in different individuals is, of course, quite great. One man

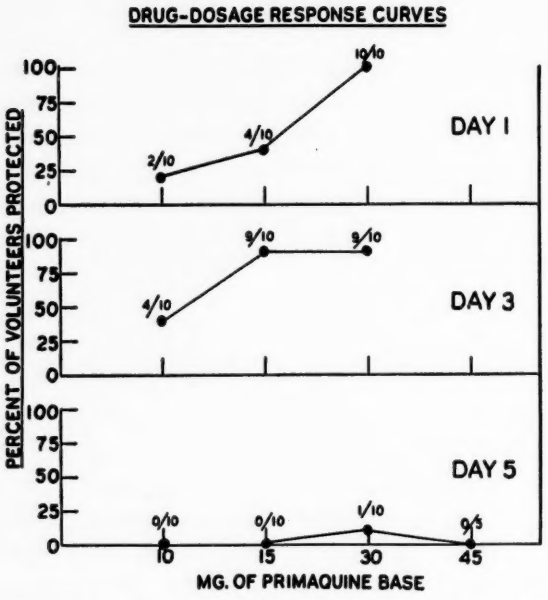

Fig. 1.—Drug-dosage response curves for primaquine given on a single day. (Ratios represent number cured over number infected.)

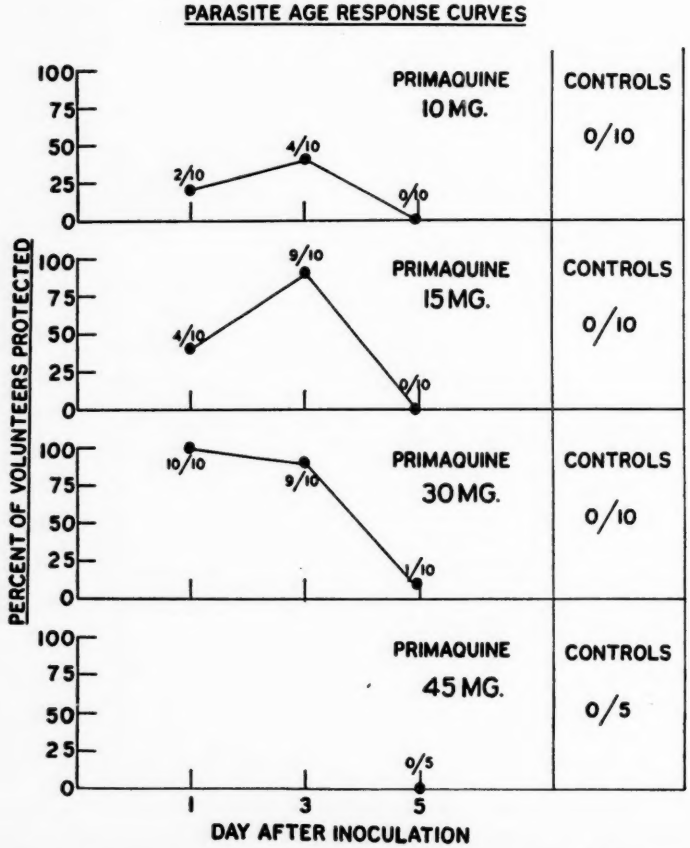

Fig. 2.—Parasite-age response curves to a given dose of primaquine. (Ratios represent number cured over number infected.

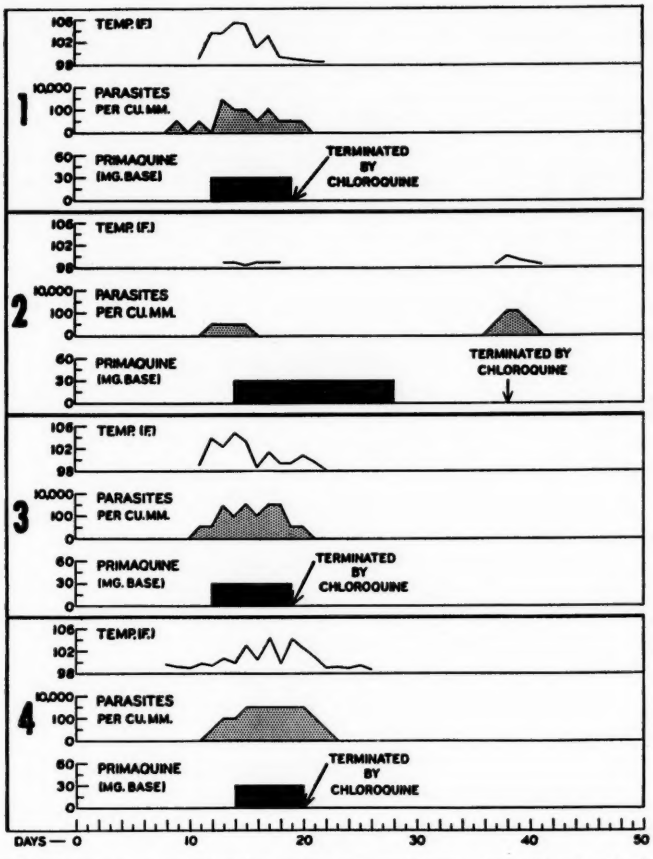

Fig. 3.—The ineffectiveness of primaquine alone as treatment for clinical attacks of falciparum malaria.

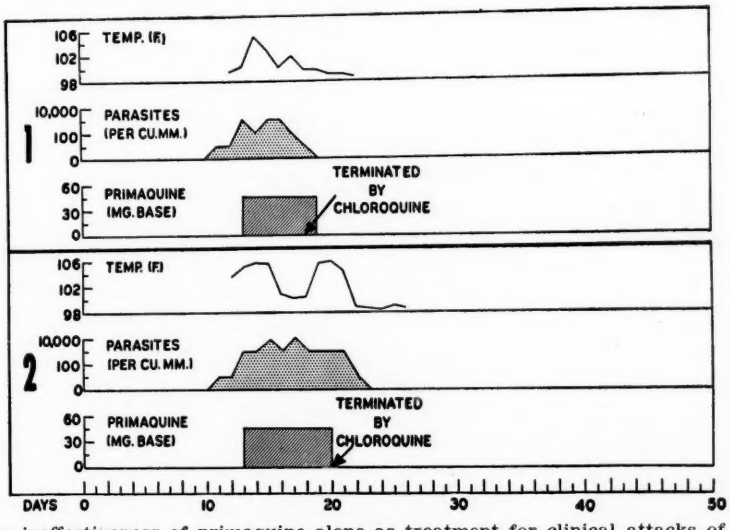

Fig. 4.—The ineffectiveness of primaquine alone as treatment for clinical attacks of falciparum malaria.

in this group (Case 2) responded well to primaquine therapy within the first three days and remained clinically well and free of circulating parasites for 10 days following the 14-day course of therapy at which time a recrudescence occurred and the patient was then treated with chloroquine.

The remaining two men were treated with 45 mg. of primaquine base (Fig. 4). In neither case did the disease respond satisfactorily to primaquine in this larger dose, and in both instances the disease had to be terminated finally by chloroquine. There did not seem to be any appreciable increase in effectiveness of primaquine in 45 mg. doses over primaquine in 30 mg. doses. This fact suggests a slowly rising dose response curve. It seems unlikely that the dose response curve could be more clearly established, because higher doses of primaquine begin to show signs of toxicity which, when added to the difficult clinical course produced by the malaria, make an unreasonable demand on the volunteer subjects.

Treatment of falciparum malaria by primaquine alone is not justified.

II. *Sexual Stages* (*Gametocyticidal Action*).—Previous studies of gametocyticidal action of other drugs in falciparum have dealt with long-term drug administration against inhomogeneous populations of gametocytes. There are several serious obstacles to devising a standardized test to give a semiquantitative measure of drug action against single broods of gametocytes in falciparum malaria. One difficulty is that the life span of any single brood of gametocytes has not been established with certainty. Another difficulty lies in the fact that nonimmune men cannot be carried easily without suppressive therapy to the period of gametocyte production. Furthermore, if trophozoite formation is not curbed and the patient has the associated chills and fever that will be produced by unchecked parasitemia he will no longer be nonimmune by the time gametocytes appear.

For these technical reasons we have as yet been unable to evaluate primaquine accurately for gametocyticidal activity.

SUMMARY AND CONCLUSIONS

It has been possible to extend the antiparasite spectrum of primaquine to several known stages of a Panamanian strain of falciparum malaria.

Primaquine has its most powerful effect on the early tissue stages of *Plasmodium falciparum*. In this it is similar to its effect on *Plasmodium vivax*. Since tissue stages of falciparum produce asexual blood forms after 144 hours post inoculation, it is possible to make this comparison only in the interval before 144 hours. Several experiments demonstrate the very intense action of primaquine against tissue stages.

The most dramatic measure of the causal prophylactic effect of primaquine was obtained by single dose treatment in the incubation period of *Plasmodium falciparum*. Ninety-five nonimmune white men were infected by a standardized technique and were then given a single test dose of primaquine (either 10, 15, 30, or 45 mg.) on one of three different days post inoculation (first, third, or fifth). When the dosage-response curve for each of the three days chosen for

treatment is considered, it is apparent that the tissue forms undergo a marked change in their response to primaquine throughout the incubation period, reaching maximum susceptibility on the third day and finally developing a highly resistant stage similar to that found in the circulating trophozoites. The data suggest that the parasite is changing biochemically so that by the fifth day it is nearly independent of the metabolic pathways reached and inhibited by therapeutic doses of primaquine.

With the asexual circulating parasites the situation has changed. The response of the parasite to primaquine has reached the extreme point of resistance. Primaquine no longer has a very substantial antimalarial effect. Once trophozoites have developed, the disease, in nonimmune white males, cannot be safely controlled in nontoxic doses. The dose-response curve then appears flat and resembles the character of the dose-response curve obtained on tissue stages on the fifth postinoculation day.

The practical lesson from this study is that primaquine should never be relied upon to control the parasitemia in nonimmunes without concurrent suppressive therapy.

Because of technical difficulties the gametocyticidal action of primaquine against single broods of gametocytes has not as yet been evaluated.

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ZONE ELECTROPHORESIS IN STUDIES OF SERUM PROTEINS, PROTEIN-BOUND POLYSACCHARIDES AND SERUM LIPIDS IN RHEUMATOID DISEASE

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AMONG the many abnormal chemical changes which occur in the course of rheumatoid disease are alterations in blood proteins. The techniques specifically applied in the past to a study of the protein disorders in these illnesses have indicated that fluctuations of certain proteins are associated with changes in clinical or laboratory status.[1] A familiar example is the association of high plasma fibrinogen levels with increases of sedimentation rate in acute and active rheumatoid disease.[2] Of the available analytical methods, electrophoresis is possessed of unique value because it is a gentle means of separation which is able to fractionate plasma into six major components and serum into five major components, and it can provide simultaneous qualitative and quantitative information relating to the separated fractions. Zone electrophoresis[3, 4] is based upon the same underlying principle and is able to separate plasma or serum into the same components. However, it is unable to provide the accurate quantitative data which are obtained when specimens are analyzed by the classical Tiselius technique.[5] It possesses the advantages of economy and simplicity of operation. Because the procedure is carried out on a solid medium, the migrated fractions may be stained selectively for proteins, carbohydrates, lipids, or other materials which may possess an affinity for a certain dye. The present paper describes experiences with the technique of zone electrophoresis in a study of serum proteins, polysaccharides, and lipids in twenty-five patients with rheumatoid arthritis and related conditions. A similar related study has been reported by Jacqueline, Groulade, and Drufovka.[6]

MATERIALS AND METHODS

Serum specimens were obtained from twenty-two patients with early acute, well-advanced acute, and chronic rheumatoid arthritis or rheumatoid spondylitis. Patients with early inflammatory disease (six months' duration or less) all gave evidence of multiple joint involvement but showed no joint deformities. Patients with advanced disease all had some objective evidence of multiple joint involvement, i.e., deformity, fibrosis, ankylosis, but differed in regard to the degree and extent of active and acute lesions. The clinical status of patients is designated numerically according to stage and functional classification using the procedure recognized by the American Rheumatism Association.[7] Practically all subjects were receiving treatment, i.e., gold, cortisone, or aspirin, or a

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combination of these at the time serum specimens were procured. Follow-up specimens were obtained in many instances so that it was possible to compare changes in serum proteins with other laboratory changes and with alterations in the clinical picture. Sera were also available from one subject with acute rheumatic fever and from two patients with lupus erythematosus disseminatus.

Zone electrophoresis on a filter paper medium was carried out using a technique described by Kunkel and Tiselius.[3] For each experiment 3 sheets of Whatman 3 mm. paper, 40 cm. in length and 14 cm. in width, were immersed in pH buffer and then dried to the barely moist state using other sheets of thick filter paper. The first sheet was placed on a silicone-treated glass plate 19 by 15 cm. in size in such a way that an equal amount of paper was free at both ends. The amount of undiluted serum used for test purposes was 0.005 ml. This was placed in the center of the paper using a special pipette. Four to eight determinations were performed at one time by appropriately spacing samples across the width of the sheet and allowing for about 2 cm. of unused paper at both sides. Bromphenol blue powder was added to one of the eight specimens so that the degree of separation of serum components during electrophoresis could be followed by observing the distance of migration of dye-stained albumin. Analyses were performed in duplicate or triplicate by superimposing one moistened piece of paper directly on top of another prepared sheet and adding the same serum samples at the same locations and then repeating the process using a third paper. A sheet of silicone-treated plastic was used to separate one serum-treated paper from another. A second 19 by 15 cm. silicone-treated glass plate was placed on top of the paper and clamps were applied to both ends and to both sides of the unit. Electrophoresis was carried out at 4° C. using barbital buffer at pH 8.6 and ionic strength 0.1. A current of from 150 to 200 volts and 15 to 20 Ma. was applied and maintained over a period of from 10 to 12 hours. Under the conditions of these experiments separations of serum into gamma, beta, alpha$_1$ and alpha$_2$ globulins, and albumin occurred over a distance of from 6 to 7 cm. After the electric current had been discontinued, each piece of paper was removed and treated separately with reagent which stained for proteins, protein-bound polysaccharides, or lipids. Protein staining was carried out using an alcoholic mixture of mercuric chloride and bromphenol blue.[3] Protein-bound polysaccharides were stained with basic fuchsin reagent following fixation and treatment with periodic acid[8-10] and serum lipids were stained with an alcoholic solution of Sudan black.[11]

RESULTS

The results of the present study are shown in Tables I, II, and III, and Figs. 1, 2, and 3. Table I summarizes the clinical and laboratory data on eight subjects with active rheumatoid arthritis. Zone electrophoresis serum patterns are stained for proteins. The existence of an inflammatory process is reflected by an increase from the normal in one or more globulins, especially the alpha and gamma globulins and to a lesser extent beta globulin. The serum patterns of Patients 1, 4, 5, and 8 in Table I indicate that globulin changes in these severely ill patients, although strikingly different from the normal, are not particularly consistent with respect to degree of increase in a given globulin component. A decrease in albumin is present in the serum of Patient 4. It was usual to find marked globulin increases early in the course of rheumatoid disease. Eight patients in whom blood studies were carried out were ill for periods of time less than six months. All of them had continued inflammatory manifestations of rheumatoid disease ranging from mild to severe. Alpha globulin increases were observed in seven, a beta globulin increase in five, and a gamma globulin increase in four subjects. Patients 1 and 2 were ill

TABLE I. ZONE ELECTROPHORESIS OF SERA FROM PATIENT WITH RHEUMATOID ARTHRITIS. ASSOCIATION WITH OTHER LABORATORY AND CLINICAL FINDINGS

Patient Number	Brief Clinical Description	Protein Pattern					Other Laboratory Data	
		Globulin				Albumin	Erythrocyte Sedimentation Rate (mm./hr. Wintrobe)	Sheep Cell Agglutination Titer (D.A.T. Waaler-Rose)
		γ	β	α_2	α_1			
1	Severe, multiple joint involvement, 4 months duration - poor response to salicylates Stage 2 Class 4						34	32
2	Mild, multiple joint involvement, 2 months Stage 1 Class 2						12	2
3	Moderate, multiple joint involvement, 11 years - ambulatory on cortisone Stage 3 Class 2						42	8
4	Severe, multiple joint involvement, 6 years - semi-invalid - gold and cortisone Stage 4 Class 3						40	32
	Normal A							
5	Severe, multiple joint involvement, 3 years - ambulatory on butazolidine and gold Stage 3 Class 2						42	16
6	Mild, mostly two or three joints involved, 6 months - ambulatory on aspirin Stage 2 Class 1						18	8
7	Moderate, multiple joint involvement, 3 years - ambulatory on aspirin Stage 2 Class 2						20	1
8	Moderate, multiple joint involvement, 24 years - ambulatory on gold and ACTH Stage 4 Class 3						38	32
				↑ Origin				

TABLE II. ABNORMAL PROTEINS IN RHEUMATOID DISEASE. ELECTROPHORETIC PATTERNS OF SERA STAINED FOR PROTEIN-BOUND POLYSACCHARIDES. ASSOCIATION WITH OTHER LABORATORY FINDINGS AND WITH CLINICAL STATUS

Patient Number	Brief Clinical Description	Protein-Bound Polysaccharides	Other Laboratory Data	
			Erythrocyte Sedimentation Rate (mm./hr. Wintrobe)	Sheep Cell Agglutination Titer (D.A.T. Waaler-Rose)
9	Moderate - multiple joint involvement, 1 year duration - bed rest - good response to cortisone and gold Stage 2 Cl.3		42	16
10	Moderate, multiple joint involvement, 3 months - bed rest - good response to salicylates Stage 1 Class 3		49	8
11	Moderate, multiple joint involvement, 5 years - ambulatory on cortisone Stage 3 Class 3		31	8
12	Moderate, multiple joint involvement, 2 months - bed rest - good response to cortisone and gold Stage 1 Class 3		30	2
13	Severe rheumatoid spondylitis, 3 years - ambulatory on cortisone and gold Stage 3 Class 3		31	16
14	Severe - multiple joint involvement, 10 years - bed rest - indifferent response to cortisone and gold Stage 4 Cl.4		34	64
15	Severe, multiple joint involvement, 11 years - bed rest - poor response to cortisone and aspirin Stage 4 Class 4		50	32
	Normal B			

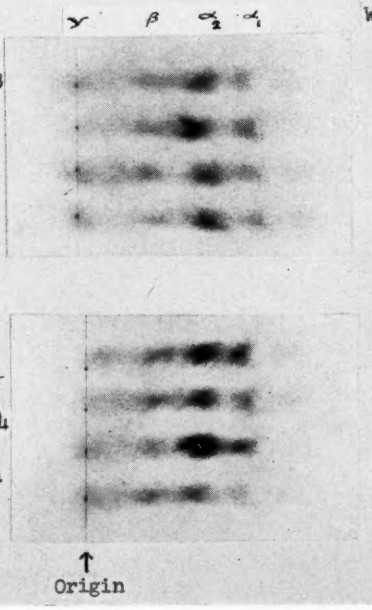
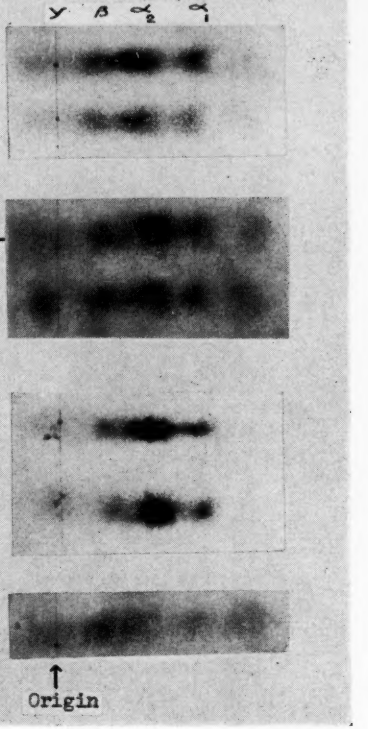


TABLE III. ZONE ELECTROPHORESIS OF SERA FROM PATIENTS WITH RHEUMATOID ARTHRITIS. FLUCTUATION IN PROTEIN-BOUND POLYSACCHARIDES ASSOCIATED WITH CHANGES IN CLINICAL STATUS

Patient Number	Date Serum Specimen was Obtained	Brief Clinical Description	Protein-Bound Polysaccharides
9	September 1954	Moderate -bed rest - Stage 2 Class 3 - treatment with cortisone and gold	
	November 1954	Ambulatory on cortisone - Stage 2 Class 2	
10	August 1954	Moderate - bed rest - Stage 1 Class 3 - erythrocyte sedimentation rate 49 - treatment with salicylates	
	November 1954	Ambulatory - Stage 1 Class 2 - erythrocyte sedimentation rate 4	
15	September 1954	Severe - semi-invalid - poor response to numerous antirheumatic drugs Stage 4 Class 4	
	November 1954	Continued severe - complete bed rest Stage 4 Class 4	
		Normal C	



four and two months, respectively. The sera from these patients show demonstrable increases in all globulin components. Fourteen patients studied were ill for periods of time ranging from six months to twenty-five years. A relatively marked gamma globulin increase was observed in eleven, beta globulin in eight, and alpha globulin in nine of these subjects. Alpha and beta globulins were normal or only mildly elevated in sera from patients with chronic illness who exhibited lesser degrees of inflammatory disease at the time blood specimens were withdrawn. It was uncommon to find sera which showed marked decreases in albumin, even in patients with severe early or long-standing disease. The serum patterns of Patients 2 through 8 are representative of these findings.

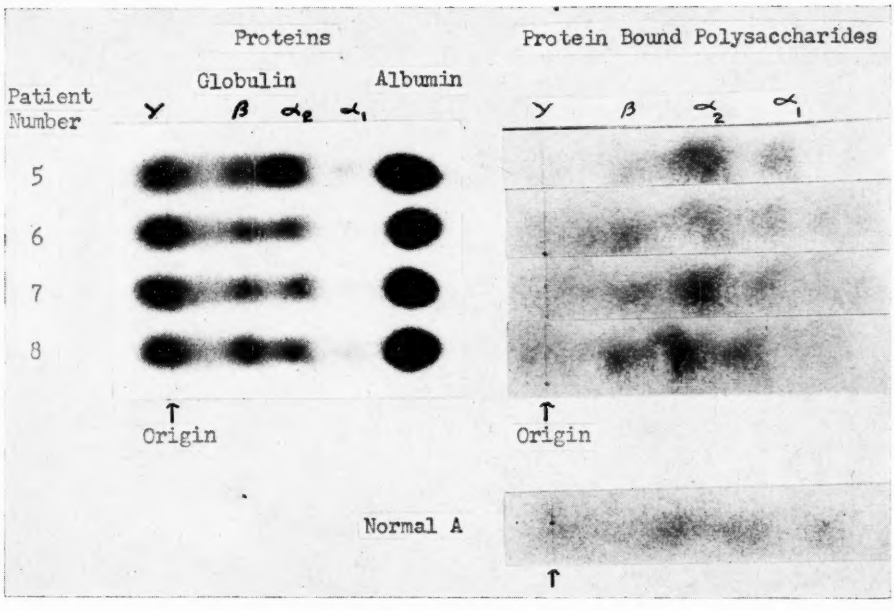


Fig. 1.—Abnormal proteins in rheumatoid arthritis. Electrophoretic patterns of sera stained for proteins and protein-bound polysaccharides.

Fig. 1 shows duplicate patterns stained for proteins and protein-bound polysaccharides in Patients 5 through 8. The intensity of disease process seemed to be well reflected by an increase in protein-bound polysaccharides as demonstrated by the periodic-fuchsin technique. The most striking increases were observed in association with the alpha$_2$ globulin, although lesser proportionate increases were associated with alpha$_1$, beta, and gamma globulins. The intensity of polysaccharide staining could usually be correlated with the severity of inflammatory disease as judged by clinical criteria (Table II). Patients 9 through 15 were all on some form of antirheumatic treatment at the time blood was withdrawn for electrophoretic analysis.

It was possible to relate a decrease in the amount of protein-bound polysaccharides to clinical improvement, as demonstrated by the response

of Patients 9 and 10 to therapy (Table III). Patient 15 showed continued and progressive severe rheumatoid arthritis and multiple inflammatory joint involvement with deformities despite intensive therapy. The serum-bound polysaccharides remained elevated during the period of time this patient was followed on therapy.

In contrast to the observed behavior of proteins and protein-bound polysaccharides, the serum lipids studied in fasting specimens from patients with rheumatoid disease were similar to lipid patterns of sera from normal subjects. There was no observable correlation between the pattern of serum lipids and changes in clinical status (Fig. 2).

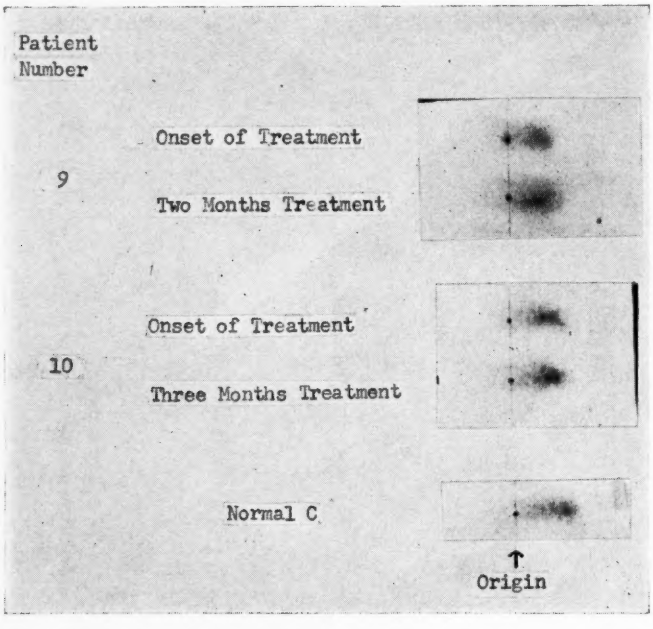


Fig. 2.—Pattern of serum lipids in rheumatoid arthritis as shown by zone electrophoresis.

When the method of electrophoresis was compared with other laboratory criteria used in following the course of rheumatoid arthritis, demonstrable increases of alpha or gamma globulins were present in Patients 2 and 7 who had mild disease with no significant elevation of erythrocyte sedimentation rate or sheep cell agglutination titer. In cases of severe inflammatory rheumatoid disease, very high sheep cell agglutination titers and sedimentation rates were always associated with marked abnormalities in serum globulins and in serum polysaccharides (Patients 1, 4, 5, and 8).

Electrophoretic patterns of sera from patients with rheumatoid arthritis were compared with patterns of sera from patients with acute rheumatic fever and disseminated lupus erythematosus. The patterns in rheumatoid arthritis and acute rheumatic fever were similar but they differed from those carried

out on patients with disseminated lupus erythematosus (Fig. 3). Serum patterns in the latter condition were associated with a preponderance of stainable material in the gamma globulin component.

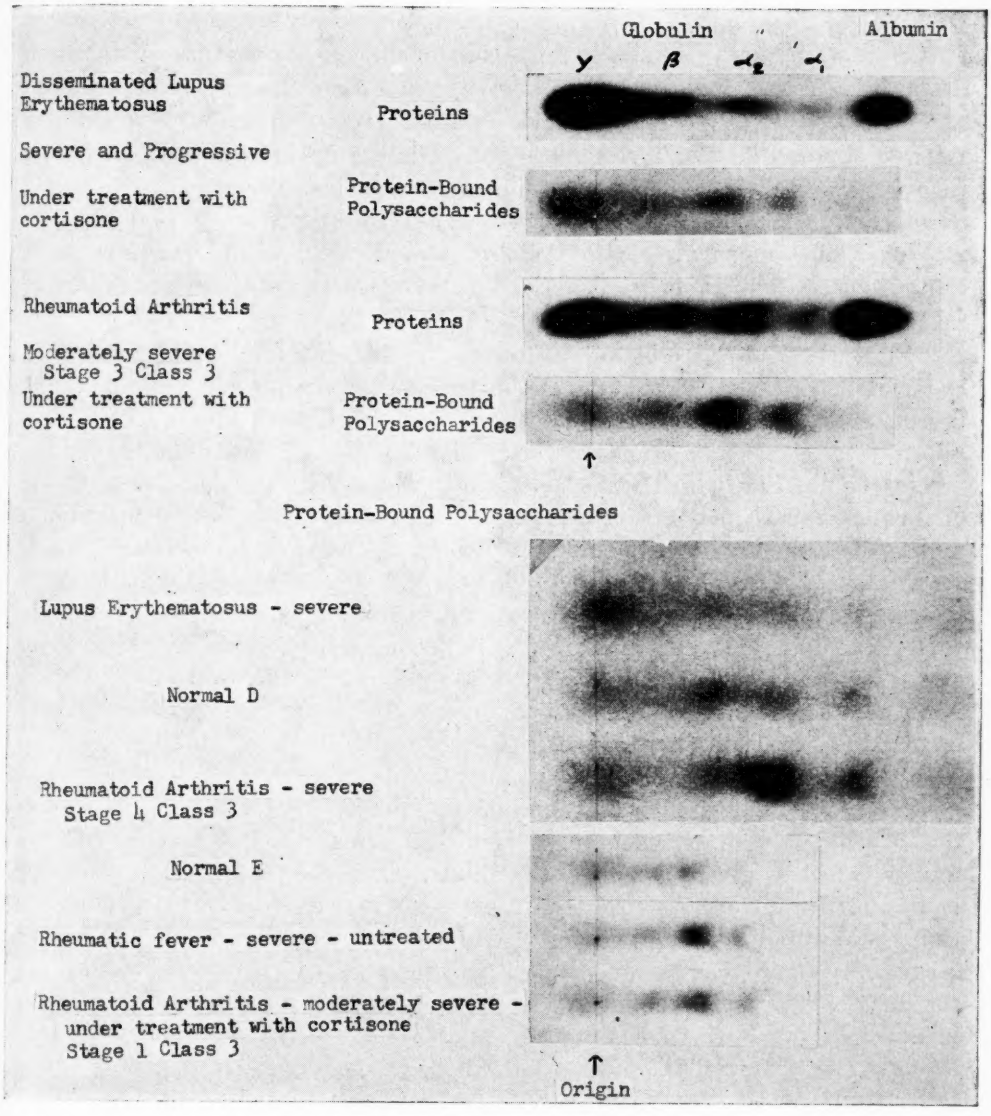


Fig. 3.—Comparison of electrophoretic patterns in rheumatoid arthritis, disseminated lupus erythematosus and rheumatic fever.

DISCUSSION

The present studies demonstrate that protein and polysaccharide components of serum, as determined by the method of zone electrophoresis, are abnormal in rheumatoid arthritis. The intensity of changes is roughly proportional to severity of the inflammatory process. The components affected

are: albumin occasionally decreased; beta globulin occasionally increased; alpha or gamma globulin generally increased. Although severely ill patients all showed marked globulin increases, there was no consistency with respect to the kind of globulin demonstrating the greatest change. These patients were on different forms of therapy at the time blood specimens were taken, a factor which may influence the behavior of certain serum components. Globulin increases were proportionately greater than a decrease in albumin and the total serum proteins remained within normal limits or were slightly increased above normal levels.[12] A good clinical response to antirheumatic therapy was associated with reversion of the electrophoretic pattern toward normal and a proportionate adjustment of the total proteins. These findings are generally comparable with those of other investigators who used zone electrophoresis and other methods of fractionation to study abnormalities of serum in rheumatoid disease.[6, 12, 13, 14, 15, 17, 18, 19, 20, 21]

Increases in protein-bound polysaccharides were found to be present in rheumatoid disease. The most commonly affected component was contained in the $alpha_2$ globulin, although other components were also affected to a lesser degree, depending upon severity of the disease. Patients with severe, active rheumatoid arthritis characteristically showed a considerable increase of protein-bound polysaccharides associated with $alpha_2$ and occasionally $alpha_1$, beta, and gamma globulins. The same components decreased in intensity associated with clinical improvement.[6, 20] It is of interest that the same kind of polysaccharide pattern is observed in sera of patients with rheumatic fever, although different in stained sera from subjects with lupus erythematosus. In the latter condition most of the polysaccharides appear to migrate with gamma globulin. When techniques other than the periodic-fuchsin method are used to study serum polysaccharides in rheumatoid disease, increases in the following components may be demonstrated: hexosamine and nonhexosamine polysaccharides and mucoproteins.[21-23] Some of these materials may be stained when the periodic-fuchsin method is carried out. A positive result is given by substances which contain the "1-2 glycol" grouping in unsubstituted form (or the equivalent form in which hydroxyl groups are replaced by amino or alkylamino groups) or its oxidation products, but in serum only the high molecular weight substances such as protein-bound polysaccharides and glycoproteins are likely to be present in adequate amounts to give visible color.

Abnormalities in electrophoretic patterns were not paralleled by other aberrant laboratory findings except when associated with severe, acute inflammatory illness. It was noticed that patients who had mild inflammatory disease with or without advanced or marked joint deformity generally had demonstrable electrophoretic anomalies even in the absence of a significant elevation of erythrocyte sedimentation rate or sheep cell agglutination titer. The fact that all patients were receiving treatment (most of them were on cortisone) at the time blood samples were withdrawn may have a bearing on these discrepancies. Kelley, Adams, and Good[24] found that treatment of

rheumatic fever with ACTH or cortisone caused deviation of elevated serum mucoprotein concentration toward normal, but that hormonal treatment had less effect on this acute phase reactant than on other indices of rheumatic activity such as erythrocyte sedimentation rate. Other workers have observed that treated cases of severe nonproliferative rheumatoid arthritis may be associated with a normal erythrocyte sedimentation rate in the presence of elevated serum globulin.[6, 25]

The method of electrophoresis as utilized in the present study is primarily a qualitative device, although the kind of information obtained may be relatively specific as demonstrated by the presence of characteristic anomalies in sera from subjects with lupus erythematosus. These findings were in contrast to those observed in rheumatoid arthritis. On the other hand, the different patterns of sera from the treated patients with rheumatoid arthritis are not peculiar to this disease, and electrophoretic analyses will not generally provide conclusive diagnostic information. However, serial patterns carried out on individual patients may be of considerable use in following the status of inflammatory lesions. Because zone electrophoresis can separate serum into its component fractions by gentle means, it is particularly well adapted as a research tool. In this realm it furnishes a new experimental approach to the detailed study of isolated and purified serum fractions from patients with rheumatoid disease in the untreated stage and under condiitons of treatment.

SUMMARY

The method of zone electrophoresis was used to fractionate sera from twenty-five patients with rheumatoid disease. Increases were observed in alpha, beta, and gamma globulin proteins, and in polysaccharides associated with all of the globulins, but mostly with the $alpha_2$ globulin. Albumin was observed to be decreased in one patient with severe manifestations of disease. Changes of serum proteins and polysaccharides toward normal occurred concomitant with improvement in clinical status. Serum lipid patterns in rheumatoid disease were not significantly different from normal lipid patterns. Electrophoretic patterns of serum from patients with disseminated lupus erythematosus did not resemble the patterns associated with rheumatoid arthritis or rheumatic fever.

Serum specimens from patients with rheumatoid disease were provided through the courtesy and cooperation of the following physicians: Drs. Harry Margolis, David Barr, Joseph Warren, Mary Garrity, and William Cooper, all from Pittsburgh, Pennsylvania. We wish to acknowledge the technical assistance of Miss Esther Ridley in the preparation of electrophoretic patterns. Photographs of patterns were prepared by the Pittsburgh Photographic Laboratory and the figures and tables were prepared by Mrs. Barbara Davidson.

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THE AMOUNT OF TRAPPED PLASMA IN THE RED CELL MASS OF THE HEMATOCRIT TUBE

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THE hematocrit reading, indicating the percentage volume occupied by the the cellular elements in whole blood, is generally considered the most reliable quantitative test in clinical laboratory medicine. Many methods of measurement of the circulating blood volume, the survival of transfused red cells and the concentration of intracellular substances depend upon the validity of the hematocrit reading. It is well known, for example, that the concentration of red cells in venous and arterial blood is 8 to 10 per cent higher than the "whole body hematocrit" when the latter is calculated on the basis of separate measurements of the circulating red cell mass and the plasma volume.[1-5] The difference between these two values has been ascribed by some investigators to the presence of "trapped plasma" in the column of packed cells measured in the hematocrit tube.[6] Others have attributed the discrepancy to an inequality in the distribution of red cells between large and small blood vessels.[7, 8]

Many attempts have been made to measure, directly or indirectly, the amount of plasma that is contained within the column of packed cells in the hematocrit tube after centrifugation. These studies are well summarized in the papers of Owen and Power[9] and Vazquez and co-workers.[10] Increments of packed cell volume attributed to trapped plasma by various authors have ranged from plus 11.7 to minus 2.4 per cent, negative values indicating that hematocrit readings in some instances were lower than the values obtained by dye, radioisotope dilution techniques, or other indirect methods. Divergent opinions continue to be published concerning the magnitude of correction factors that should be applied to observed hematocrit readings in order to compensate for the apparent additive effect of trapped plasma. Owen and Vazquez[9, 10] postulated that between 2.1 and 2.9 per cent of the "observed" cell mass in centrifuged blood samples is comprised of trapped plasma. Chapin and Ross,[6] on the basis of dye dilution experiments, considered that observed hematocrit values are erroneously high by a factor of 8 per cent and routinely applied a correction factor of 0.92 to all of their hematocrit readings. Hlad and Holmes,[11] on the other hand, stated that it is impossible to correct hematocrit readings for trapped plasma since the amount of trapping varies from sample to sample, depending on the character of the specimen and the length of the hematocrit cell column. Chaplin and Mollison[1] have concluded

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that the amount of trapping varies in accordance with the height of the packed cell column and that the correlation is sufficiently close to permit correction of hematocrit readings over a wide range. Most of the experiments that have been reported to date with the exception of those described by Chaplin and Mollison have been limited to studies on samples having hematocrit readings which lie within the normal range.

The present study has been designed to determine the degree to which plasma is trapped in the packed cell mass in samples with abnormal as well as normal hematocrit readings. Mixtures of red cells and plasma were prepared with cell concentrations ranging from approximately 18 to 75 per cent. The cell concentration in each sample was then determined by two different methods: (1) by measuring the volume of packed cells in samples subjected to a centrifugal force equal to 2,000 × G for 60 minutes in Wintrobe or Gibson hematocrit tubes; (2) by calculating the volume of extracellular fluid in the sample (that is, the plasmatocrit) from the degree to which Cr^{51}-labeled hemoglobin was diluted when added in constant proportions to mixtures of cells and plasma. Hemoglobin was selected as the solute in these dilution studies because this substance neither permeates the cell membrane nor alters its physiologic characteristics. Cr^{51} was employed as a tagging agent in view of the powerful affinity that has been shown to exist between chromium and hemoglobin,[13] the combination being sufficiently stable to preclude the possibility that the tag might later become disassociated and recombine thereafter with hemoglobin or with some other component of the intact red cells.

METHODS

Preparation of Tagged Hemoglobin Solution.—Sodium chromate^{51} solution, containing 0.1 to 0.3 mg. of chromium metal and with an activity equivalent to 100 to 150 μc was added to approximately 35 ml. of Group 0 whole blood collected in 8 ml. of acid-citrate-dextrose solution (ACD). This mixture was allowed to stand at room temperature for approximately one hour, after which the red cells were separated by centrifugation and washed 4 times with approximately 50 ml. of isotonic saline solution. A test of the supernatant solution after the fourth washing indicated the presence of less radioactivity than twice background, between 80 and 90 per cent of the added sodium chromate having firmly combined with the red cell mass. Next, the red cells were hemolyzed by alternate freezing and thawing in carbon dioxide snow and in a 37° C. water bath. The hemolysate was then diluted with 5 volumes of isotonic saline solution in order to reduce its viscosity and facilitate its quantitative transfer. In order to ensure the absence of any residual uncombined sodium chromate which might be contained in the hemolysate, the latter was then combined with 15 ml. of washed red cells obtained from fresh Group AB blood. This mixture was allowed to stand for one hour at room temperature, after which the cells were removed by sedimentation at 2,000 x G for one hour. Sterile precautions were observed in the preparation of this hemolysate and the material was stored prior to use at 4° to 6° C. in order to prevent deterioration from bacterial contamination. The absence of insoluble radioactive material which might be sedimented together with the red cells in test preparations was further ensured by passing the hemolysate through a syringe filter prior to its addition to the test specimens. At the conclusion of each hemoglobin dilution experiment, the red cells were washed 4 times in saline solution and their radioactivity was measured by a scintillation counter. Radioactivity was invariably absent, indicating that no radioactivity had been lost from the plasma as a result of sedmentation or absorption on red cells in these preparations.

Preparation of Test Samples.—Forty to sixty milliliter samples of venous blood were collected from healthy young men and women and placed in flasks containing 2 ml. of a solution containing ammonium oxalate, 0.12 Gm., potassium oxalate, 0.4 Gm., and NaCl, 0.43 Gm. Each sample was rotated on a 22 cm. radius at 2,000 r.p.m. for 20 minutes, after which the supernatant plasma was removed. The buffy coat was likewise removed as completely as possible and discarded. The plasma and red cells were then recombined in proportions designed to prepare 15 to 25 ml. of a suspension containing red cells in approximately the desired concentration. One to two milliliters of hemoglobin solution labeled with radioactive sodium chromate were added through a syringe Seitz filter. The preparation was subjected to vigorous agitation for at least two minutes in order to ensure thorough mixing preparatory to sampling.

Direct Hematocrit Readings.—Aliquot samples of each test specimen were placed in three Wintrobe or Gibson hematocrit tubes. These were rotated at 3,000 r.p.m. for 60 minutes on a 20 cm. radius. The radius was measured from the middle of the rotating head to the distal end of the centrifuge tube. The total volume of packed cells, including the buffy coat, was then measured, and the average value calculated in per cent was recorded in Table I. The standard error obtained in triplicate hematocrit readings in this experiment was calculated to be 0.81 per cent.

Isotope Dilution Technique.—One or two milliliter aliquot samples were delivered into each of three test tubes, the same volumetric pipette being used for all transfers. After each test tube had received its sample of blood, the pipette was rinsed 3 times with saline solution. All rinse solution was collected into the test tube. The final volume of the test specimen was adjusted to 5 ml. by adding distilled water. The remainder of the sample of the whole blood-hemoglobin mixture was then centrifuged at 2,000 r.p.m. for one-half hour on a 20 cm. radius and the supernatant plasma removed. Three 1 to 2 ml. samples of this material were transferred into test tubes. The same pipette previously used to deliver the whole blood was also used for this procedure, after having been washed thoroughly with a detergent, rinsed many times with distilled water, and dried with alcohol and ether. The pipette was rinsed with saline solution to ensure complete delivery of the contents into the test tube in each case. The viscosity of whole blood is greater than that of plasma and, had this procedure not been done, it is possible that less whole blood would actually have been delivered than plasma.

The radioactivity of the whole blood and plasma samples was measured by a well-type scintillation counter* with a sodium iodinethallium activated crystal. The background ranged between 108 to 112 counts per minute (CPM); 1 μc of Cr^{51} was equal to 80,000 CPM. The results were obtained in counts per minute per aliquot sample and a sufficient number of counts were taken to keep a standard deviation due to counting errors alone well below 1 per cent. The plasmatocrit of the whole blood sample as measured by isotopic dilution was determined from the ratio of whole blood and plasma radioactivity, as follows:

$$\text{plasmatocrit} = \frac{\text{CPM contained in an aliquot of whole blood} \times 100}{\text{CPM contained in an aliquot of plasma}}$$

$$\text{cellcrit} = 100 - \text{plasmatocrit.}$$

The reproducibility of the cell volume data obtained in this manner is indicated by the fact that the average value for one standard deviation from the mean cellcrit, based on triplicate determinations performed on 52 samples, was 3.0 per cent.

RESULTS

The per cent difference between the hematocrit reading and the cell volume, as calculated from the dilution of chromated hemoglobin relative to the concentration of red cells representing "trapped plasma," was determined in 52 plasma–red cell mixtures. The amount of plasma contained in the sedimented portion of these samples after centrifugation, as shown in Table I, was 1.3 ± 0.41 per cent (mean ± standard error of the mean) of the packed cell mass.

*Atomic Instrument Company.

TABLE I

EXPERIMENT NUMBER	CELL VOLUME (PER CENT) (A) HEMATOCRIT READING	CELL VOLUME (PER CENT) (B) DILUTION OF Cr^{51} TAGGED HEMOGLOBIN	PERCENTILE DIFFERENCE BETWEEN VALUES A AND B $\frac{(A - B \times 100)}{A}$
1	18.8	18.8	0.0
2	23.6	23.8	-0.8
3	23.7	23.0	3.0
4	24.3	24.4	-0.4
5	25.0	24.7	1.2
6	25.1	26.7	-6.4
7	27.1	28.3	-4.4
8	27.5	26.1	5.1
9	27.5	28.1	-2.2
10	28.3	29.6	-4.6
11	30.0	32.2	-7.3
12	31.4	35.3	-12.4
13	31.7	31.5	0.6
14	33.9	33.3	1.8
15	35.3	35.0	0.8
16	35.6	35.8	-0.6
17	36.7	36.6	0.3
18	38.6	36.0	6.7
19	40.8	39.9	2.2
20	41.0	40.0	2.4
21	41.6	40.0	3.8
22	41.7	40.4	3.1
23	42.1	42.0	0.2
24	42.3	41.9	0.9
25	42.6	42.4	0.5
26	42.7	40.9	4.2
27	42.9	42.2	1.6
28	45.4	46.1	-1.5
29	45.6	45.1	1.1
30	45.8	46.0	-0.4
31	49.3	47.1	4.5
32	49.7	47.1	5.2
33	50.3	48.5	1.2
34	50.4	49.9	1.0
35	50.5	47.8	5.3
36	50.8	48.9	3.7
37	52.1	52.0	0.2
38	53.2	52.2	1.9
39	53.3	52.4	1.7
40	54.0	52.1	3.5
41	54.7	54.0	1.3
42	60.5	58.7	3.0
43	60.8	59.0	3.0
44	62.8	61.2	2.5
45	63.4	56.9	10.2
46	65.1	64.0	1.7
47	65.4	63.9	2.3
48	67.4	65.7	2.5
49	67.9	66.1	2.7
50	70.4	68.2	3.1
51	73.0	69.8	4.4
52	75.5	73.3	2.9
Mean ± S. E. of the mean entire series			1.3 ± .41

The relation between degree of "plasma trapping" and cellcrit was subjected to statistical analysis according to the method described by A. B. Hill.[14] The average of all the results obtained in these experiments is represented by a straight line determined by the regression equation $y = 0.12x - 3.95$.* The correlation coefficient expressing this relationship was computed to be 0.49 and the standard error of r, 0.14. Since the ratio of r to the standard error of r is greater than 3, it is apparent that a significant positive correlation must exist between the volume of packed cells, or the height of the cell column after centrifugation, and the volume of plasma that is included within the latter and contributes to its height. This relationship is illustrated in Fig. 1, in which plasma trapping (y), expressed in terms of percentage of the red cell volume, is plotted in relation to the hematocrit reading (x).

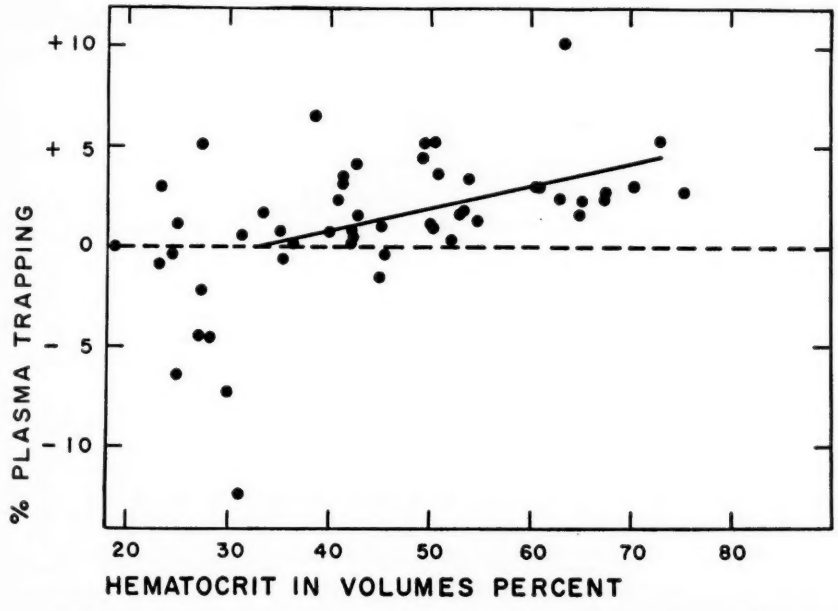

Fig. 1.—The amount of plasma in the red cell column, expressed as per cent plasma trapped versus the hematocrit reading. The regression equation for the solid line is given in the text.

DISCUSSION

The amount of plasma trapped in the cell column of the hematocrit tube has been determined in samples of experimentally constituted anemic, normal, and erythremic blood. These measurements have been based on the difference between the hematocrit reading, that is, the volume of packed cells relative to

*Where x equals the cellcrit, and y = trapped plasma (per cent), and r = the coefficient of correlation between x and y

$$r = \frac{\Sigma (x-\bar{x})\ (y-\bar{y})}{\sigma x \ \sigma y \ n}$$

$$y-\bar{y} = r \frac{\sigma y}{\sigma x}(x-\bar{x})$$

$$\sigma \text{ of } r = \frac{1}{\sqrt{n-1}}$$

the total blood volume, and a value representing cell volume, in per cent, which was derived from the ratio of whole blood and plasma radioactivity in aliquot samples diluted with Cr^{51}-labeled hemoglobin solution. The difference between the observed and the calculated hematocrit values, divided by the observed value and multiplied by 100, is assumed to represent "plasma trapping," or the percentage error in the hematocrit reading that is attributable to the inclusion of plasma. Relatively small variations in the direct hematocrit readings or in the cell volumes determined by isotopic dilution produce very substantial changes in the apparent "plasma trapping." For example, if the calculated hematocrit value were 40.0 and the observed value 40.8, plasma trapping would be considered 2 per cent. However, if it were assumed that the hematocrit reading is erroneous, the true value being 41.2 per cent, then the trapped plasma would be computed as 2.9 per cent. Thus, a 1 per cent error in the hematocrit reading would have led to a 45 per cent error in the calculation of plasma trapping. An extensive discussion of statistical errors involved in studies of this type has been published by Vazquez and associates.[10]

Direct measurements of trapped plasma, although less vulnerable to statistical error, pose technical problems which outweigh those involved in the indirect method. Owen[9] and Hlad and Holmes[11] have reported that trapped plasma is not evenly distributed throughout the red cell mass. Hlad and Holmes[11] have made direct measurements of plasma in cell columns by means of a complex system of external scanning through a 1 mm. slit and also have sampled these columns directly through the bottoms of Lusteroid tubes. They concluded that plasma is trapped in progressively greater amounts toward the top of a packed cell mass. It follows that, unless the entire cell column can be removed intact from the centrifuged sample, its analysis for plasma content will be subject to gross error. Vazquez and co-workers[10] have attempted to aspirate the cell mass in toto with a pipette, but were confronted with the impossibility of draining this glassware clean and were obliged to apply a correction factor to compensate for the loss of viscous cell mass which clung in significant amount to the walls of this pipette. Furthermore, it is almost impossible to aspirate the top portion of the cell column without including plasma. These criticisms do not appear to apply to the work of Chaplin, Mollison and Vetter[1] who by the direct method found progressively more plasma trapped as the height of the cell column increased.

Data obtained in the present studies indicate that a positive correlation exists between the quantity of plasma trapped and the height of the red cell column. The data indicate that 0.8 per cent plasma or less was included in the cell column of hematocrit samples with readings less than 40 per cent; 2 per cent when the reading was 50 per cent, and 4.0 per cent for a hematocrit of 68 per cent. Our data for these hematocrit ranges are in close agreement with that of Chaplin and Mollison,[12] who found 2, 2.5, and 3 per cent plasma trapping, respectively, for hematocrits of 40, 50, and 68 per cent. These authors did find significant amounts of plasma trapping in bloods with hematocrit reading

below 30 volumes per cent. From our data, it is impossible to conclude that any measurable quantity of plasma is trapped in samples with hematocrit readings of 33 per cent or below.

SUMMARY AND CONCLUSION

The cell volume in 52 blood samples with hematocrit readings ranging from 18.7 to 75.4 per cent has been measured directly by hematocrit readings and indirectly computed from the dilution of chromium^{51}-labeled hemoglobin solution. The difference between these values is considered to represent the volume of plasma "trapped" in the packed cell mass of centrifuged samples of blood. The data demonstrate a direct correlation between the height of the red cell column in the hematocrit tube and the amount of plasma trapped for blood samples with hematocrit readings of from 33 to 73 per cent. For blood samples with hematocrit readings of 33 per cent or less, no plasma trapping could be demonstrated.

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ANTIHYPERTENSIVE EFFECTS OF METAL BINDING AGENTS*

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ALL known agents which are antihypertensive in man and animals and which do not block autonomic nerves, such as thiocyanate,[1] nitroprusside,[2] azide,[3] hydrazinophthalazine,[4] 2,3-dimercaptopropanol,[5] and other mercaptans and thiohydrazines,[6] are characterized by an affinity for certain trace metals. To assess the role of metal binding, chelators of unknown vasoactivity were tested; some were found to lower blood pressure in anesthetized renal-hypertensive but not normotensive rats. To limit those metals which might be involved, a series of disodium ethylenediamine tetra-acetates of increasingly tightly bound divalent metals was assayed for depressor activity in both types of animals. Both the relatively acute changes fifteen minutes after a single dose and the cumulative effects one to three hours after several spaced injections of the same substance were observed. To evaluate the possibility of dissociation or cationic excess in the chelate solution, small amounts of the free metal ions were used as controls. In addition, certain metals complexed to organic compounds were evaluated.

METHOD

Three hundred and forty-three Sherman rats weighing 220 to 270 grams were anesthetized with 12 to 15 mg. of intraperitoneal pentobarbital sodium, tracheotomized, and connected to a Sanborn electromanometer by a needle inserted into one femoral artery. A cannula was then placed in the contralateral femoral vein for introduction of test material. The dose of 5 mg. for each chelator or metal ethylenediamine tetra-acetate and 0.2 mg. for each metal dichloride was dissolved in 0.5 ml. saline and neutralized to pH 7.4 ± 0.1. Before, during, and after injection the blood pressure was directly recorded graphically. Further tracings were made 5, 10, 15, and 20 minutes later. At intervals of about 40 minutes successive similar doses of a substance were given to measure cumulative effects. Experiments usually lasted from one to three hours, and no more than one compound was given to any rat. One renal artery of 179 animals had been partially constricted three to five weeks previously, and hypertension was present at the time of the experiment (diastolic pressure more than 100 mm. Hg in each case, mean 119 mm.). The other 164 animals were not operated upon and were normotensive (diastolic pressure always less than 100 mm. Hg, mean 77 mm.). From two to twelve normotensive and from three to eight hypertensive rats were used to assay each substance. Only responses sustained for at least 15 minutes were considered of interest.

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In order to evaluate random variations in diastolic pressure under the conditions of the experiment, 0.50 ml. of isotonic sodium chloride at pH 7.4 was injected intravenously into 85 unoperated rats with diastolic pressures from 60 to 100 mm. Hg. The average diastolic fall at 5 minutes was 0.0 ± 13.9* mm. Hg; at 10 minutes it was 4.6 ± 13.7 mm.; at 20 minutes it was 1.8 ± 14.4 mm. In 100 operated rats with diastolic pressures over 100 mm. Hg the changes were similar, at 5, 10, and 20 minutes the mean diastolic depressions being 1.1 ± 15.4, 4.8 ± 19.7, and 5.3 ± 14.8 mm. Hg, respectively.

Because of the large number of substances assayed and the small number of injections of any one substance into a particular kind of animal, no rigorous statistical treatment of data was attempted. There was about one chance in twenty that an isolated diastolic depression of 30 to 35 mm. Hg was fortuitous. When unidirectional changes in all animals for each substance were analyzed, the spread of the data was such that a mean diastolic fall for a group of values (i.e., 3 to 10 at a certain time after one substance was given either to hypertensive or normotensive animals) had at least the same significance when it exceeded 20 mm. Hg.

In order to ascertain whether or not the various substances depressed the reactivity of vascular smooth muscle to vasoconstrictor agents, a standard dose of norepinephrine (0.3γ) was injected intravenously before, and several times after, the unknown was given. At least 30 minutes were allowed to elapse between unknown and norepinephrine. Diastolic elevations were compared with the controls.

RESULTS

Various Chelators.—Fifteen minutes after injection, 5 mg. of 1-hydrazinophthalazine (hydralazine) was significantly vasodepressor for hypertensive rats, and there was no essential difference in its effect 1 to 2 hours later after a total dose of 15 mg. There was a moderate depressor effect in normotensive animals, both soon after an injection, and at the end of the experiment, which under the criteria established were questionably significant. In hypertensive animals the cumulative effect of 8-hydroxyquinoline sulfonic acid was similar but less marked and the acute effect was of questionable significance. There was no vasodepression in normotensive animals, in fact, quite the contrary (Table I). In hypertensive animals the cumulative effect of Perma Kleer,† a

TABLE I. CHANGES IN MEAN DIASTOLIC PRESSURE AFTER METAL CHELATORS

SUBSTANCE	NO. RATS*	MEAN CHANGE IN DIASTOLIC PRESSURE, MM. HG			
		ACUTE (15 MIN.+)		CUMULATIVE (60-150 MIN.+)	
		NORMO-TENSIVE	HYPER-TENSIVE	NORMO-TENSIVE	HYPER-TENSIVE
Hydralazine	10/18	–22	*–49*	–22	*–56*
8-hydroxyquinoline sulfonic acid	4/9	+24	–22	+7	*–35*
Perma Kleer	2/7	+3	–8	0	*–29*
Sopanox	5/10	+34	+52	–9	–10
$Na_2CaEDTA$	8/13	–1	–13	–8	*–42*

*The first figure represents the number of normotensive, the second the total number of animals.

Figures in italics are considered significant. Cumulative refers to the change after subsequent injections, acute being those after the first.

EDTA = ethylenediamine tetra-acetate.

*The values in this paragraph are expressed as the mean plus or minus the standard deviation.

†Supplied by Dr. F. B. Zienty of Monsanto Chemical Company, St. Louis, Mo.

polyaminocarboxylic acid, was similar but less, and the substance was inert in normal rats. Sopanox,* 1-σ-tolylbiguanide, a weaker chelating agent, had no cumulative action but was acutely pressor irrespective of blood pressure, reminiscent of the acute elevation of normal blood pressure caused by 8-hydroxyquinoline sulfonic acid in rats and by 2,3-dimercaptopropanol in man.[5]

Metal Ethylenediamine Tetra-acetates.†—The calcium, chromous, manganous, cobaltous, and possibly the nickelous disodium ethylenediamine tetra-acetates (sequestrenates) produced significant cumulative vasodepressant effects in hypertensive rats (Fig. 1). The inert chelates of the more loosely bound divalent metals were the magnesium and the dihydrogen disodium sequestrenates, which alone of the series could directly affect calcium or magnesium concentrations in body fluids. Of the 10 analogues none significantly altered normal blood pressure. Table II lists these compounds in the order of increasingly tight linkage with the metals; the larger the log K_2, the less dissociation between cation and chelator. Only the first three compounds are capable of altering normal alkaline earth or alkali metal concentrations; for the more tightly bound chelates no disturbance of normal electrolyte patterns clouds the interpretation of their effects. The one trivalent metal complex tested, ferric, was pressor in normotensive rats. No significant alteration in pressor response to a standard (0.3γ) dose of intravenous norepinephrine was observed after any of the chelates or chelating agents. A representative experiment is shown in Fig. 2.

Metal Salts.—To exclude a metallic effect from partial dissociation of the metal chelate, small amounts of the dichlorides of chromium, manganese, iron, cobalt, nickel, copper, zinc, and cadmium were similarly assayed, as well as the trichlorides of iron and chromium, and pentavalent vanadium. Three of these eleven metals had significant acute or cumulative vasoactivity within 20 minutes of injection, vanadium being pressor and zinc and chromic ions being depressor. When the second injection was given, however, ferrous, cobaltous, cupric, and zinc selectively lowered the diastolic pressure of hypertensive animals, affecting normotensive ones less or not at all (Fig. 1). The other five metals failed to produce delayed or selective effects. Significant differences between the actions of metal ions and chelates are shown in Table II.

Complexes of Metals With Pyridoxal and Amino Acids.—On the possibility that an organic chelate of a metal might be active, certain pyridoxylidenemetal amino complexes‡ were injected into rats in 2.5 mg. doses as outlined above. The series of compounds does not parallel all of the metals used as sequestrenates, since these complexes are difficult to make and many were unavailable. Table III shows the results. Definite selective vasoactivity was observed by one complex of each metal but zinc, although no consistent effects appeared relating to the metal or the amino compound.

*Supplied by Dr. F. B. Zienty of Monsanto Chemical Company, St. Louis, Mo.
†Supplied by Mr. H. W. Zussman of Geigy Industrial Chemicals, New York.
‡Synthesized and provided by Dr. Karl Folkers, Merck & Company, Inc.

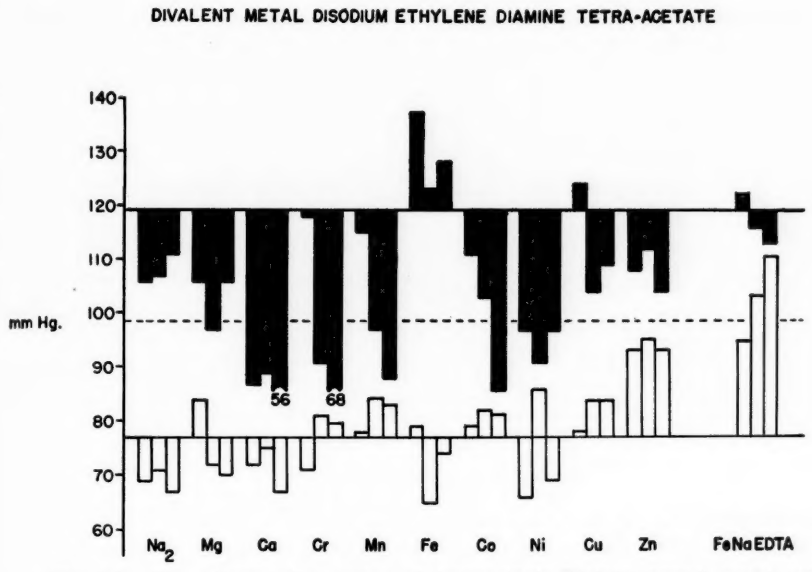

Fig. 1*A*.—The effects of a series of increasingly tightly bound metal ethylenediamine tetra-acetates on the diastolic pressures of groups of hypertensive (upper bars) and normotensive (lower bars) anesthetized rats. The first bar for each metal complex represents the change 20 to 30 minutes after one intravenous injection of 5 mg.; the second and third changes a like interval after subsequent injections. Mean changes are shown, each group representing at least 3 and usually 4 or more rats. All complexes were dihydrogen metal except for ferric as shown on the right. Note the comparable differences in hypertensive (mean diastolic pressure 119 mm. Hg) and normotensive animals (mean diastolic pressure 77 mm. Hg) as well as the small effects with sodium and magnesium complexes, which can alter calcium levels in blood, and the decreased effects with nickel, copper, zinc and trivalent iron. Metals are listed in order of atomic number.

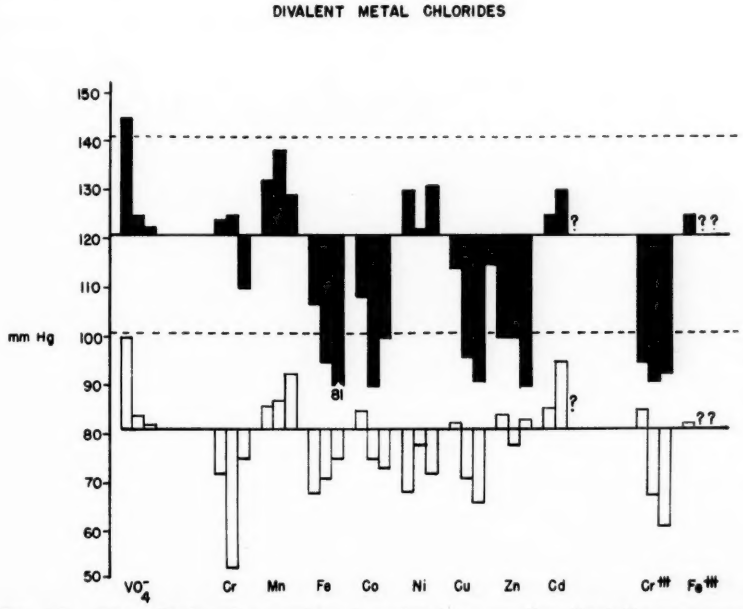

Fig. 1*B*.—The effects of a series of metal ions on the diastolic pressures of groups of hypertensive (mean diastolic pressure 121 mm. Hg) and normotensive (mean diastolic pressure 81 mm. Hg) anesthetized rats similarly treated. Doses were 0.2 mg. For obvious reasons sodium and calcium salts were not given. The pressor action of pervanadate is indicated as well as are the effects of cadmium and chromic chlorides. Note the differences between the ions and their complexes as shown in *A*, especially in regard to chromous, manganous, ferrous, nickelous, zinc, and ferric iron which was inert in large doses.

TABLE II. SIGNIFICANT EFFECTS ON DIASTOLIC BLOOD PRESSURE OF METAL CHELATES (15 MG.) AND ION (0.6 MG.) IN HYPERTENSIVE RATS

Me^{++}	ATOMIC NO.	LOG K_2	$H_2MeEDTA$ (MM. Hg)	$MeCl_2$ (MM. Hg)
Na_2	11	1.66	--	--
Mg	12	8.69	--	--
Ca	20	10.59	-63	--
Cr	24	13.00	-51	--
Mn	25	13.47	-31	--
Fe	26	14.22	--	-40
Co	27	16.10	-33	(-21)
Zn	30	16.58	--	-29
Ni	28	18.45	(-22)	--
Cu	29	18.38	--	-28
$Fe^{+++}Na$		25.00	+33	--

Log K_2 is an index of the stability of the chelate, the higher values being more stable. The figures were taken from *Sequestrene*, a publication of the Alrose Chemical Co., Providence, quoting Schwarzenbach and associates. That for chromous is not exactly known.

EDTA = ethylenediamine tetra-acetate.

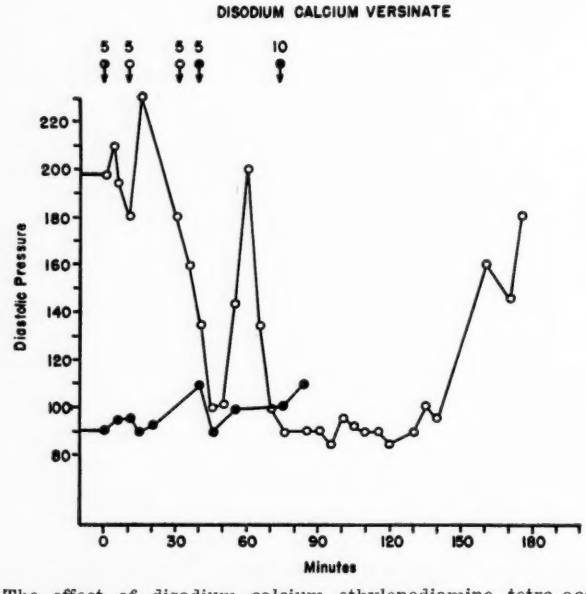

Fig. 2.—The effect of disodium calcium ethylenediamine tetra-acetate on the diastolic pressure of anesthetized rats. Open circles: renal hypertensive animal; closed circles: normotensive animal. The doses in milligrams are shown, representing that given to each rat. Note the lack of effect on the normotensive of 20 mg., while 15 mg. was temporarily depressor in the severely hypertensive animal. A Sanborn electromanometer recorded blood pressure. The rises in diastolic pressure induced by norepinephrine were: normal, control 62, at 90 minutes, 58; hypertensive, 45, at 130 minutes, 55 mm. Hg. Note that one animal was severely hypertensive; in all other cases the effects persisted as long as the experiment was continued.

DISCUSSION

The data presented are consistent with the following hypothesis. Free chelators and loosely associated metal chelates bound one or more trace metal ions in the bodies of rats with a resultant antihypertensive effect. As the association between the chelate and the different metals increased, it eventually became sufficiently tight that the active endogenous metal was no longer bound. Such chelates were inert. The predicted log K_2 value for ethylenediamine tetra-acetate of such an endogenous metal is slightly more than 16 since this

value corresponds to the change between an antihypertensive effect and vasoinactivity. Since the transition and near-by metals are bound by several antihypertensive agents as well as by ethylenediamine tetra-acetate, it is logical to search among them for a possible exogenous or endogenous metal as being concerned with vasospasm. The tightness of the bond between most chelates and divalent metals of or near the first transition series increases regularly with atomic weight, reaching a maximum at copper and then falling off sharply with zinc.[7] The dissociation constants for trivalent ions are less systematized, but in general these ions are bound in similar order but more tightly than their divalent counterparts.

TABLE III. SELECTIVE DEPRESSOR EFFECTS OF PYRIDOXAL-METAL-AMINO COMPLEXES ON BLOOD PRESSURE OF RATS

PYRIDOXAL—PLUS / PLUS	Co	Zn	Ni	Al	Cu
Glutamic acid	0	0			0
Phenylalanine	±		0	+	
Phenethylamine	0				0
Arginine			+		0
Glycine				0	
Tyrosine					+
Isobutylamine					0

+ indicates mean depression of > 25 mm. of hypertensive diastolic pressure with > 20 mm. difference between hypertensive and normotensive.

± indicates mean depression of 20 to 25 mm. of hypertensive diastolic pressure with > 20 mm. difference between hypertensive and normotensive.

0 indicates insignificant changes, or pressor responses. From four to six pairs of rats were used for each compound.

Eliminating the sodium and magnesium chelates because they bind calcium and magnesium, and the nickel chelate because of its equivocal effect, it is evident that the calcium, chromous, manganous, and cobaltous chelates with a log K_2 value of less than 16.2 were active and that the cupric, zinc, and ferric chelates with higher log K_2 values were inert. The only exception was the ferrous chelate. The instability of this valence state of iron with respect to the trivalent form plus the very much tighter binding of ferric as compared with ferrous chelate suggests that partial oxidation may have occurred.

Whenever one metal is liberated from a chelator, a like amount of another cation must be bound. Since the total amount of manganese, cobalt, iron, copper, and zinc in extracellular fluids amounts to approximately 300γ per cent, not much metal would be released from a chelate if all of them were bound. To control a direct action of a liberated metal, the metal chlorides themselves were given, for it is known, for example, that cobalt ion is a vasodilator[4b, 8] and vanadium a vasoconstrictor substance.[9] It is important to our concept that chromous and manganous ions were not depressant although their chelates were, so that for the first pair of transition metal chelates, at least, the antihypertensive effect was not due to liberation of the injected metal. Protein flocculation may have been produced by some of the other ions, causing depression of blood pressure. This reaction was avoided by using metals tightly bound to pyridoxal in equivalent amounts.

It is possible that certain metalloenzymes are involved in vascular abnormalities. Both renal decarboxylase of dihydroxyphenyl alanine (DOPA) and monamine oxidase are affected by certain trace metals of the first transition group.[10] Hepatic synthesis of cholesterol and fatty acids is profoundly altered by some of the same metals.[11] Metal binding agents affect all three systems,[10, 12] either by enhancing or depressing activity. The specific element in hypertensive rats removed or activated by chelating agents is, however, unknown.

SUMMARY

Intravenous injections of the three most powerful metal binding agents of four tested exhibited antihypertensive activities in anesthetized rats. A correlation was established between the dissociation constant of the metals chelated with ethylenediamine tetra-acetate and their antihypertensive activities, tightly bound compounds being inert. Small amounts of some of the same metal ions themselves were inactive while others differed in their effects. Metals tightly bound to another organic substance further differed in their vascular activities.

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PYREXIN, THE PYROGENIC FACTOR OF INFLAMMATORY EXUDATES, AND ITS RELATION TO SOME BACTERIAL PYROGENS*

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INTRODUCTION

IN 1943, I showed that the euglobulin fraction of acid exudates, in particular, has pyrogenic activity.[1] Subsequently a factor was extracted from this fraction and shown to be capable of inducing an elevation in temperature in both rabbits and in dogs. This factor was termed pyrexin.[2] The evidence at the time indicated that this pyrogenic factor was probably a peptide. In 1952, the factor was crystallized from an acetic acid mother liquor, and further observations substantiated the possible peptide character of this factor.[3] Pyrexin is heat stable, and its presence in acid exudates was believed to offer a reasonable explanation for the primary mechanism of fever with acute inflammation. Several years ago Bennett[4] confirmed the presence of a febrile factor in exudates. This investigator[4] and Holderman[5] concluded that the substance in sterile exudates capable of causing fever in rabbits is not a bacterial pyrogen. Recently, however, Bennett and Beeson[6] have reported on the existence of a relatively thermolabile pyrogenic substance in association with granulocytes. They have also shown that rabbits acquire a tolerance to small doses of pyrexin (0.25 mg.). These investigators, therefore, have questioned the view that pyrexin can reasonably be envisaged as concerned with the mechanism of fever with inflammation owing to its tolerance-inducing property. Furthermore, Bennett and Beeson[6] have pointed out that there is a possibility that the pyrexin I describe is a contaminant with some bacterial pyrogen acquired during the chemical extractive procedures. Widström and Almgren[7] have recently observed that sterile exudates from cases of acute pleurisy induce fever when injected intravenously into rabbits. They found that transudates from patients with uncompensated heart failure yield negative pyrogenic effects. These workers have also found that in some cases of acute pleurisy in which there seemed to be considerable pyrogenic activity in the exudate, there was found likewise evidence of activity in the blood plasma. I have previously reported that ascitic fluid does not seem to contain the pyrogenic factor,[8] and at times concomitantly with an acute inflammation I have observed pyrogenic activity in the blood serum.[2]

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The problem of tolerance to bacterial pyrogens has been considered by several writers. Beeson[9] is of the opinion that consequent to a series of injections of a pyrogen, there follows a change in the functional capacity of the reticulo-endothelial system, whereby there is an enhancement in the ability to remove bacterial toxins from the blood, thus protecting other susceptible tissues. Grant and Whalen[10] have shown a diminution of the latent period following the injection of blood withdrawn from a donor previously injected with a bacterial pyrogen. This effect is believed by them to be due to the formation of an "endogenous pyrogen" by the interaction of the bacterial pyrogen and plasma.[11] Subsequently Grant[11] expressed the view that refractoriness involves a loss of capacity to form the "endogenous pyrogen." More recently Grant has postulated the presence of a plasma "inhibitor" as one important factor in the development of refractoriness to pyrogens.[12] Farr and Lequire[13, 14] have observed that a pyrogen mixed and incubated with serum or plasma from a normal rabbit induced a greater fever response than when the same pyrogen was incubated with saline. Farr and his collaborators[15] have recently confirmed the original observations; but in addition they have shown that the serum of tolerant rabbits when incubated with a pyrogen fails to enhance the fever response. They attribute their findings to the presence in appreciable amounts of a gamma globulin inhibitor which is antagonistic to the fever-augmenting effects of normal serum. Contrary to the earlier observations of Beeson,[9] these investigators found that a passive transfer of a mild degree of tolerance can be achieved.[15] The observations of Farr and Lequire[13, 14] on the potentiating effect of serum on pyrogens are of great interest. They are reminiscent of early experiments by Mondolfo and Hounie[16] who found that *Escherichia coli* grown in serum produced more pyrogen than in common broth cultures. On the other hand, Hegemann[19] more recently has reported that the highly potent lipopolysaccharide of Westphal[17, 18] is inactivated by human plasma.

Pyrexin as originally extracted from exudative material induces, besides an elevation in temperature, an initial leukopenia and a subsequent leukocytosis.[1, 2] The leukopenia was found to be referable to a leukopenic factor which could readily be dissociated by partial hydrolysis from crude pyrexin.[20] The development of a delayed leukocytosis following the administration of pyrexin was ascribed to a thermostable leukocytosis factor.[21] This leukocytosis-inducing component was crystallized and as such found free of any pyrogenic property.[22] These biological characteristics of amorphous or crude pyrexin are precisely the same properties displayed by bacterial pyrogens, namely, elevation in temperature, initial leukopenia, and subsequent leukocytosis. The analogous response caused by these different pyrogenic substances prompted an investigation in an endeavor to determine whether there may not exist some common denominator. The present work indicates that the intravenous injection in rabbits of some bacterial pyrogens possibly converts the euglobulin of blood plasma into a material possessing the gross biological properties of pyrexin as extracted from exudates. However, I do not venture to state

definitely, at least at this stage, exactly how the bacterial pyrogens induce the formation of the pyrogenic euglobulin. In other words, no positive claim can be made as to whether the pyrogen actually converts the euglobulin into a pyrexin-like compound or whether the pyrogen merely unites with the gamma globulin to form a pyrogen-globulin complex. The significant point in the present study seems to be that the euglobulin portion of the plasma proteins is the selected fraction for either combination or conversion. Furthermore, the present studies indicate that with repeated doses of pyrexin roughly comparable to the amount of pyrexin recovered from inflammatory exudates, there is no development of tolerance to this substance. Finally, the evidence indicates little likelihood that pyrexin is a pyrogen contaminant during the extractive procedures.

MATERIALS, METHODS, AND EXPERIMENTAL RESULTS

The Failure To Induce Tolerance With Large Doses of Pyrexin.—Experiments were set up to determine whether rabbits became tolerant to repeated administration of pyrexin in doses approximating the amount of that substance recovered from inflammatory exudates. The recovery of pyrexin from exudates varies considerably. It tends to exist in larger amounts in acid exudates than in alkaline exudates. Several determinations of pyrexin by the method previously described[2] yielded values ranging from 1.2 to at least 9.8 mg. per milliliter of exudate. In acid exudates the concentration of this substance is at times even of a very much higher magnitude. Bennett and Beeson[6] had found that repeated injections of pyrexin into rabbits for 9 days in minute doses of 0.25 mg. per

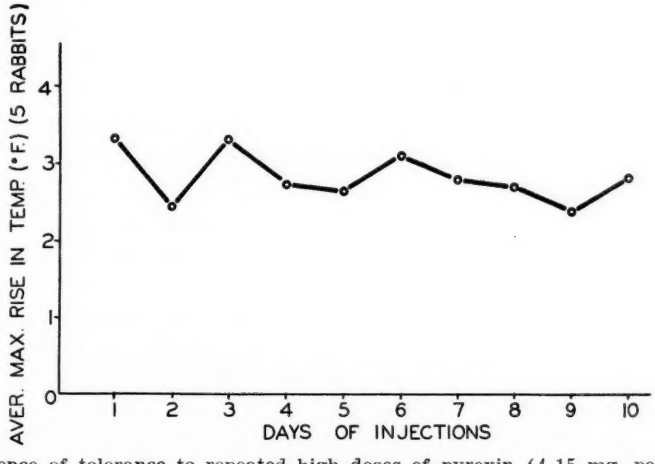

Fig. 1.—Absence of tolerance to repeated high doses of pyrexin (4-15 mg. per injection) over a period of 10 days.

injection induced a definite state of tolerance. Since this represents an amount considerably below the amount of pyrexin recovered per milliliter of exudative material, the question of acquired tolerance to pyrexin was reinvestigated by utilizing definitely higher doses of this pyrogenic substance. A series of rabbits were repeatedly injected with pyrexin in 0.5 ml. of physiological saline in concentrations ranging from 4 to 15 mg. The injections were repeated almost daily for a period of 10 days. The results obtained indicated that with the concentrations of pyrexin utilized, in contrast to the findings of Bennett and Beeson[6] with lower doses, there was found no evidence of any acquired tolerance to pyrexin. The doses of pyrexin employed were more of the order of magnitude

of the recovered amount per milliliter of exudates than the dose utilized in the experiments of Bennett and Beeson.[6] A graph showing the average daily change in temperature upon repeated administration of pyrexin is illustrated in Fig. 1. Tolerance was not evaluated merely by the maximum temperature elicited, but also by the duration during which the fever is maintained. This was found in all cases in the interval studied (about 4 hours) not to differ essentially after several daily injections of pyrexin from that found following the initial injection of this pyrogenic substance.

In view of the absence of tolerance to pyrexin with the high doses of the material, there seems no reason for discarding the view that pyrexin is one of the primary causes of fever with inflammation.

Is pyrexin a contaminant by a bacterial pyrogen?—This possibility was recently raised by Bennett and Beeson[6] and subsequently restated by Atkins and Wood.[30] This does not seem to be a valid criticism for the following reasons:

1. Pyrexin obtained by exactly the same chemical method, but from alkaline exudates instead of acid exudates, is usually inactive.[23]

2. The leukocytosis-promoting factor (LPF) and necrosin, when properly extracted from exudates, are pyrogenically inactive.[2, 8] The *initial methods* of extracting these substances from exudates are the same as used for pyrexin.[24] If pyrexin was referable to a contamination, it is doubtful that the specificity of that pyrogenic factor would be as sharp as observed, and that the other factors would not likewise be found to be pyrogenic.

3. Pyrexin extracted from blood serum is inactive.[2] Yet precisely the same method of extraction was used as when pyrexin is recovered from exudates.

4. Distilled water was used in the extraction procedure; the physiological saline and tap water utilized were all found to be nonpyrogenic.

5. The recovered pyrexin crystals from an acetic acid mother liquor were found pyrogenic, whereas the mother liquor was observed to be definitely less pyrogenic.[3]

6. Pyrexin obtained from minced canine tissue by similar methods[2] as used for exudates is, however, inactive. This was the case with samples of spleen, liver, and kidney tissues.

7. When necrosin is inactive pyrogenically in a sample of exudate, pyrexin is usually very potent, and vice versa. This indicates, as previously reported, that the pyrogenic factor is at times incapable of being readily dissociated from necrosin except by standing on ice.[25] These facts argue against the possibility of a contaminant of these substances by a bacterial pyrogen, for otherwise the pyrogenic factor would more likely be distributed quite homogeneously between these two substances.

8. All of the glassware used in the chemical extraction of pyrexin were sterilized previously to their utilization by dry heat at 170°-190° C. for 2 to 3 hours. It is true that it is somewhat difficult to obtain, in the thoracic cavity, a sterile exudate in spite of the precautions employed. This may be due to the penetration of microorganisms through the respiratory tract in damaged pulmonary tissue. The organisms were found, as a rule, to be cocci and not gram-negative organisms, the chief source, in turn, of rapidly acting

bacterial pyrogens. In brief, in view of the previously mentioned considerations, and of the specificity of pyrexin, in contrast to the other chemical factors derived from inflammatory exudates, it is quite improbable that contamination with pyrogens is a factor in the activity of pyrexin.

Furthermore, various observations indicate that some of the other factors extracted from inflammatory exudates (e.g., leukotaxine, the LPF, and necrosin) are incapable, in contrast to pyrexin, of inducing an appreciable elevation of temperature in rabbits. As stated before, some of these substances (e.g., the LPF and necrosin) are likewise derived from exudates by similar *initial* extractive methods as utilized in the extraction of pyrexin.[24] Yet, in contrast to the specificity of pyrexin they are essentially nonpyrogenic. All these facts strongly indicate that one is not dealing in pyrexin with a contaminant by bacterial pyrogens during the extractive procedure of this substance. Pyrexin has always been considered an endogenous pyrogenic substance liberated by severely injured cells at the site of inflammation.

Effect of Intravenous Injection of Bacterial Pyrogens on Euglobulin of Serum.—As I have already stated, sufficiently large doses of pyrexin, i.e., of the order of magnitude encountered in exudates, fail to induce any tolerance to this substance following its repeated injections into rabbits (Fig. 1). Furthermore, that pyrexin may be a contaminant by a bacterial pyrogen is quite unlikely in view of the evidence just presented. Yet amorphous or crude pyrexin derived from exudates and bacterial pyrogens have precisely the same type of biological properties. This consists of inducing fever, an initial leukopenia, and a subsequent leukocytosis. Is there a common denominator that can perhaps reasonably explain these facts?

Pyrexin is a component of the euglobulin fraction of inflammatory exudates.[2] Studies were undertaken to determine whether bacterial pyrogens introduced into the circulation have any effect on the euglobulin of blood serum. Two types of pyrogens were utilized, namely Westphal's[17] lipopolysaccharide from *E. coli* and Shear's[26] polysaccharide from *Serratia marcescens.** Several concentrations of Westphal's lipopolysaccharide ranging from 0.6 to 3.0 mg. and suspended in 0.5 ml. of physiological saline were injected intravenously into rabbits. There followed within a half to over one hour a relatively marked elevation in temperature, varying from 2° to almost 3° F. Two rabbits were also injected intravenously with the polysaccharide of *S. marcescens* (prepared by Dr. Shear) in concentrations ranging from 2 to 4 mg. Within two hours there followed with this pyrogen an elevation in temperature of almost 2° F. Twelve minutes to slightly over three hours after the injection of either of these pyrogens into the circulation, the animals were bled 25 to 30 ml. from the heart. Sudden death sometimes followed soon after cardiac puncture. With the large doses of the pyrogen utilized, death also ensued at times several hours after the exsanguination.

The serum was collected and a part was subsequently fractionated at one-third saturation with $(NH_4)_2SO_4$. The supernatant phase containing the

*I am indebted to Dr. Shear and Dr. Westphal for supplying these pyrogens.

pseudoglobulin (alpha and beta globulins)-albumin fraction was thereafter dialyzed against water. The euglobulin (primarily gamma globulin) precipitate was treated precisely by the same procedure that had been originally adopted for the extraction of pyrexin from the euglobulin fraction of inflammatory exudates.[2] This consisted in the addition of distilled water to the euglobulin precipitate in an amount equivalent to the withdrawn supernatant phase. Gentle stirring caused the precipitate to enter readily into solution. The dissolved euglobulin was dialyzed against tap water in a cellophane tube until free of sulfate ions. At the end of dialysis a precipitate was present in suspension throughout the length of the cellophane tube, but more abundantly at the bottom of the tube. This material (containing the precipitate sus-

TABLE I. THE MAXIMUM TEMPERATURE RISE WITHIN 4 HOURS IN DIFFERENT RABBITS CAUSED BY VARIOUS PROTEIN FRACTIONS OF BLOOD SERUM FOLLOWING THE INTRAVENOUS INJECTION OF A BACTERIAL PYROGEN*

INJECTED RABBIT FROM WHICH BLOOD SERUM DERIVED	AMOUNT AND MATERIAL INJECTED INTRAVENOUSLY MG.	INTERVAL BETWEEN INJECTION OF PYROGEN AND BLEEDING OF RABBIT HR.: MIN.	WHOLE SERUM ° F.	EUGLOBULIN (GAMMA GLOBULIN) ° F.	PSEUDOGLOBULIN-ALBUMIN ° F.	"GLOBULINS" (PRIMARILY ALPHA GLOBULINS) ° F.	NO. OF EXPERIMENTS
21-90	2.2 (L.p.)†	1:56		4.1 (whole)	1.8	0.8	7
				3.2 (ppt.)	1.2	1.1	
				4.4 (supernatant)			
21-98	3.0 (L.p.)	1:17		4.5 (whole)		1.0	7
				3.9 (ppt.)	0.7	1.6	
				2.5 (supernatant)	1.8		
21-70	2.1 (L.p.)	0:15		3.2 (whole)	1.6		5
				3.6 (ppt.)	0.6		
				4.2 (supernatant)			
22-40	0.6 (L.p.)	0:22	2.4§	2.4 (whole)	2.0	0.8	8
			1.3‖	3.2 (ppt.)	2.2		
				2.3 (supernatant)			
21-86	1.1 (L.p.)	0:12	5.0	3.4 (whole)	3.5	0	10
			2.9	4.0 (ppt.)	1.4	1.0	
				3.7 (supernatant)		0	
22-59	2-3 (S.)‡	3:11	2.4	2.6 (whole)	1.0	0.4	13
			1.7	2.4 (ppt.)	0.6	0.2	
			1.8	1.1 (supernatant)	1.0 decr.		
				2.4 (ppt.)			
				1.6 (supernatant)			
22-60	4 (S.)	0:20	4.0	3.2 (whole)	1.0	0.9	13
			2.6	2.7 (ppt.)	0.6	0.9	
				1.8 (supernatant)			
			3.3	2.42 (ppt.)	1.3		
				1.0 (supernatant)			
Average			2.7	3.3° F. (whole euglobulin)	1.3	0.7	Total of 63 experiments
				3.1° F. (precipitate)			
				2.5° F. (supernatant)			

*Every temperature rise recorded on the table represents a separate experiment on an injected rabbit and studied for a period of about 4 hours.

†L.p. refers to Westphal's lipopolysaccharide from *E. coli*.

‡S. refers to Shear's polysaccharide from *S. marcescens*.

§0.45 c.c. of serum used.

‖0.20 c.c. of serum injected.

pended in fluid) was considered as the "whole euglobulin" fraction of the serum. An aliquot 0.5 ml. sample injected into a rabbit indicated that it was highly pyrogenic to rabbits. As indicated in Table I, it induced within at least 4 hours or less a maximum elevation in temperature ranging from 2.4° to 4.5° F. with an average of 3.3° F. The rest of the euglobulin fraction was refrigerated overnight at approximately 2° C. This transferring to a low temperature favors the spontaneous separation of both a supernatant and a sediment or precipitate phase. The same can be obtained equally well by merely centrifugation of the "whole euglobulin" fraction. The separation was done in order to determine, if any, the predilection of pyrogenic activity. As shown in Table I, both of the fractions of the euglobulin are markedly pyrogenic. In two out of nine observations the precipitate seemed to be somewhat more pyrogenic than the supernatant phase. The precipitate induced an average rise of 3.1° F. and the supernatant 2.5° F.

The pseudoglobulin-albumin fraction following dialysis was found considerably less pyrogenic than the euglobulins (Table I). The average of 16 observations induced a maximum average increase of 1.3° F. A part of the pseudoglobulin-albumin fraction was treated with $(NH_4)_2SO_4$ at one-half saturation and refrigerated for several days. The clear albumin supernatant was siphoned off. The precipitate, consisting primarily of alpha globulins and some beta globulins, was centrifuged at relatively low speed (2,000 r.p.m. for 5 minutes). The cloudy supernatant phase consisting primarily of alpha globulins was dialyzed against tap water. These globulins were assayed in rabbits and found to be essentially nonpyrogenic. The average of 12 observations showed a rise of 0.7° F.

Whole serum, generally in amounts of 0.5 ml., injected intravenously, induced an average rise in 10 experiments of 2.7° F. As already summarized, and as tabulated in 63 experiments (Table I), the pyrogenic activity of blood serum following the introduction of either of the two bacterial pyrogens seems to be located primarily in the euglobulin fraction. This fraction is Biuret and Molisch positive, suggesting perhaps the possibility of a carbohydrate linkage to this nitrogenous fraction. In any event, the bacterial pyrogens injected in the rabbit fail to induce a homogeneous distribution of pyrogenic activity in the various protein fractions of blood plasma. In this connection, it is to be recalled that pyrexin is likewise a component of the euglobulin fraction of inflammatory exudates.

Effect of in Vitro Incubation of Bacterial Pyrogens and Serum, or Some Plasma Protein Fractions, on Pyrogenic Activity.—Experiments were set up to determine whether the incubation of rabbit blood serum or its derived protein fractions, with a bacterial pyrogen likewise induces the localization of pyrogenic activity in the euglobulin fraction of serum. Samples of rabbit serum ranging from 7.5 to 14 ml. were incubated at 37°-38° C. with either 0.7 mg. of Shear's polysaccharide from *S. marcescens* or 100 μg of Westphal's lipopolysaccharide from *E. coli* for periods of either 40 minutes or 2 hours. An aliquot of this incubated serum was assayed on a rabbit and found to be actively pyrogenic. Nine observations yielded an average rise of 2.3° F.

The incubated blood serum was fractionated, as described, for the euglobulin (gamma globulin), the pseudoglobulin-albumin (beta globulins, alpha globulins, and albumin) and primarily the alpha globulins. All of these fractions were assayed in a total of 60 experiments, listed in Table II. The re-

TABLE II. THE MAXIMUM TEMPERATURE RISE WITHIN 4 HOURS IN DIFFERENT RABBITS CAUSED BY VARIOUS PROTEIN FRACTIONS OF BLOOD SERUM FOLLOWING THE IN VITRO INCUBATION OF THAT RABBIT SERUM WITH A BACTERIAL PYROGEN

RABBIT NO. FROM WHICH SERUM OBTAINED	AMOUNT OF SERUM AND TYPE OF PYROGEN EMPLOYED	DURATION OF INCUBATION AT 37° C. HR.: MIN.	WHOLE SERUM ° F.	EUGLOBULIN (GAMMA GLOBULIN) ° F.	PSEUDOGLOBULIN-ALBUMIN ° F.	"GLOBULINS" (PRIMARILY ALPHA GLOBULINS) °F.	NO. OF EXPERIMENTS
22-61	7.5 ml. serum + 0.7 mg. (S.)*	2:00		3.4 (whole)	3.7	4.6	12
				2.6 (ppt.)	3.4	3.3	
				4.0 (supernatant)			
				4.8 (ppt.)	2.5		
				2.8 (supernatant)			
				3.2 (ppt.)			
				2.2 (supernatant)			
22-71	8.0 ml. serum + 0.7 mg. (S.)	2:00	2.2	2.6 (whole)	4.2	4.1	14
			3.2	2.6 (ppt.)	2.4	3.4	
				3.2 (supernatant)		3.4	
				4.0 (ppt.)	3.0	3.1	
				1.7 (supernatant)			
21-62	12 ml. serum + 100 μ (L.p.)†	0:40	1.9	2.5 (whole)	3.8	0.6	18
			1.4	0.8 (ppt.)	2.8	1.0	
			2.5	2.2 (supernatant)			
			2.1	2.9 (ppt.)	2.6	1.0‡	
				1.7 (supernatant)	2.8		
				1.9 (ppt.)			
				3.4 (supernatant)			
22-78	14 ml. serum + 100 μ (L.p.)†	2:0	2.2	4.2 (ppt.)	2.3		16
				2.3 (supernatant)			
			2.3	3.5 (ppt.)	0.5		
				2.8 (supernatant)			
			2.6		1.7	2.1	
					2.7	2.6	
				1.6 (ppt.)§		1.6	
				2.5 (supernatant)			
Average	-	-	2.3	2.8° F. (whole) euglobulin	2.7	2.6	60 experiments
				2.9° F. (precipitate)			
				2.6° F. (supernatant)			

*(S.) refers to Shear's polysaccharide from *S. marcescens.*
†(L.p.) refers to Westphal's lipopolysaccharide.
‡1 ml. injected instead of 0.5 ml.
§Only 0.25 ml. of euglobulin precipitate injected.

sults indicate, in contrast to the in vivo experiments (Table I), that the pyrogenic activity tends to be equally partitioned in the 3 fractions studied (Table II). The whole euglobulin induced an average rise of 2.8° F., the pseudoglobulin-albumin, an average elevation of 2.7° F., and the alpha globulins an average increment of 2.6° F. These results when compared with the observations assembled in Table I suggest that the bacterial pyrogens utilized in vivo localize pyrogenic activity primarily in the euglobulin fraction of serum. It therefore seems that the presence of viable cells or possibly enzymes in the circulation, readily destroyed in vitro, are a requisite for the possible formation by pyrogens of an active euglobulin in the circulation.

A criticism may be raised that the relative effective concentration of the pyrogens utilized in the in vitro experiments is greater than that used in the in vivo experiments. This is difficult to estimate in view of the fact that in the rabbit one is dealing with a blood volume of 200-300 ml. and therefore the final dilution of the injected pyrogen cannot be determined with any accuracy. Also, the question as to whether all the injected bacterial pyrogen remains in the circulation cannot be determined. Nevertheless, the utilization of two different bacterial pyrogens in the in vitro experiments at varying concentrations (0.7 mg. of Shear's polysaccharide and 100 μg of Westphal's lipopolysaccharide) have failed to alter the ultimate effects (Table II). For this reason, it is believed that if excessive concentration of the pyrogen in the in vitro experiments is the chief factor in the inability to bind sufficient pyrogen to the euglobulin, the varying concentrations (100 to 700 μg) of the two pyrogens utilized would probably yield different results. The observations, however, with the two pyrogens have yielded in the in vitro experiments essentially similar effects (Table II). The mere incubation of the bacterial pyrogens studied in physiological saline leaves their pyrogenic capacity essentially unaltered.

The Effect of Pyrexin on the Pyrogenic Activity of Various Protein Fractions of Blood Serum.—With the observation that bacterial pyrogens transform, upon their intravenous injection, the euglobulin fraction of serum into an active pyrogenic factor, the question arose concerning the effect of injections of pyrexin on blood serum and its fractions. Rabbits were injected intravenously with varying amounts of pyrexin ranging from 1.4 to 9.9 mg. From 14 minutes to one hour and 16 minutes following the administration of pyrexin, the animal was bled, as described, by cardiac puncture. Five-tenths milliliter of the prepared blood serum was tested and found devoid of pyrogenic activity (Table III). The average of 3 observations yielded an increase of 0.3° F. The serum was then fractionated as outlined. In a total of 33 experimental observations, the three fractions studied showed no pyrogenic activity (Table III). The whole euglobulin induced an average rise of 0.08° F.; the pseudoglobulin-albumin fraction caused an average increase of 0.32° F., and the alpha globulins induced essentially no change in temperature (Table III). It appears as if, in contrast to the bacterial pyrogens studied, pyrexin induces fever without in turn altering the activity of blood proteins.

TABLE III. TEMPERATURE CHANGE WITHIN 4 HOURS IN DIFFERENT RABBITS CAUSED BY VARIOUS PROTEIN FRACTIONS OF BLOOD SERUM FOLLOWING THE INTRAVENOUS INJECTION OF PYREXIN

INJECTED RABBIT FROM WHICH BLOOD SERUM DERIVED	AMOUNT OF PYREXIN ADMINISTERED MG.	INTERVAL BETWEEN INJECTION OF PYREXIN AND BLEEDING OF RABBIT HR.:MIN.	WHOLE SERUM ° F.	EUGLOBULIN (GAMMA GLOBULIN) ° F.	PSEUDOGLOBULIN-ALBUMIN ° F.	"GLOBULINS" (PRIMARILY ALPHA GLOBULINS) ° F.	NO. OF EXPERIMENTS
21-91	6.5	1:16		+0.4 (whole) +0.6 (ppt.) +0.8 (supernatant)	+1.9 +0.9* +0.8	+0.1 -0.2	8
22-27	7.3	0:18		-0.1 (whole) +1.0 (ppt.) +0.6 (supernatant)	-0.1 -0.4		5
21-75	9.1	0:14		-0.1 (whole) -0.5 (ppt.) 0.0 (supernatant)	0.0 0.0		5
21-99	9.9	0:15	-0.6	-0.3 (whole) -0.6 (ppt.)* -0.6 (supernatant)*	-0.5 -0.3*		6
22-54	1.4	1:05	+1.1 +0.4	+0.5 (whole) 0.0 (ppt.) +0.1 (supernatant)	+0.5 +0.7	+0.5 -0.4	9
Average	6.84 mg.	-	0.3	+0.08° F. (whole euglobulin)	+0.32	0.0	33 experiments

*Temperature recorded only for a period of 2 to 3 hours.

TABLE IV. SUMMARY OF THE EXPERIMENTS WITH SOME OF THE PROTEIN FRACTIONS OF BLOOD SERUM OF RABBITS TREATED WITH PYROGENS OR WITH PYREXIN

MATERIAL INJECTED AND METHOD OF ADMINISTRATION	MAXIMUM INDUCED RISE IN TEMPERATURE (° F.)					
	EUGLOBULIN (GAMMA GLOBULIN)			PSEUDOGLOBULIN-ALBUMIN	"GLOBULINS" (PRIMARILY ALPHA GLOBULINS)	NO. OF EXPERIMENTS
	WHOLE FRACTION	PRECIPITATE PART	SUPERNATANT PART			
Pyrexin (intravenously)	0.8° F.	0.1° F.	0.2° F.	0.3° F.	0.0° F.	33
Pyrogen *a.* Westphal's lipopolysaccharide *b.* Shear's polysaccharide (intravenously)	3.3° F.	3.1° F.	2.5° F.	1.3° F.	0.7° F.	63
Pyrogen *a.* Westphal's lipopolysaccharide *b.* Shear's polysaccharide (in vitro incubation with serum)	2.8° F.	2.9° F.	2.6° F.	2.7° F.	2.6° F.	60

In conclusion, the lipopolysaccharide of Westphal and the polysaccharide of Shear induce a fever response by affecting primarily the euglobulin of blood serum. The in vitro incubation of these pyrogens with serum does not favor the localization of the pyrogenic activity in any of the plasma proteins,

but rather the activity is fairly well distributed throughout all the fractions. Pyrexin, on the other hand, has no significant effect on any of the plasma proteins. These results are conveniently summarized in Table IV. The observations are also shown graphically in Fig. 2.

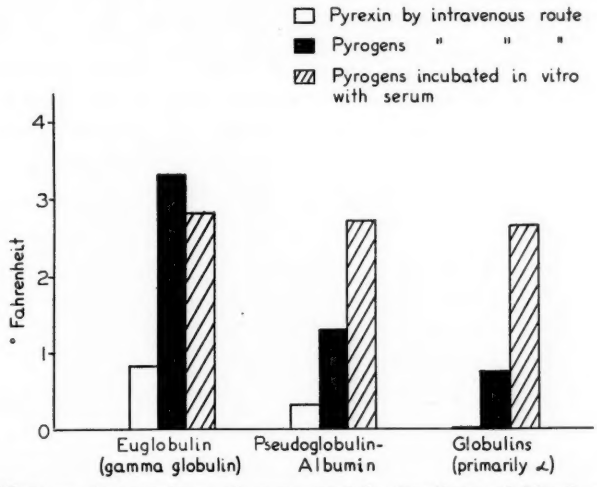

Fig. 2.—Effect on temperature of some protein fractions of blood serum following administration of either pyrexin or some bacterial pyrogens. Note that pyrexin had no significant effect on the plasma proteins. The intravenous injection of bacterial pyrogens induced a pronounced effect on the pyrogenic activity of the euglobulin fraction of serum; while in vitro incubation of the bacterial pyrogens with blood serum induced pyrogenic activity in all of the three plasma protein fractions.

The Effect of the Pyrogenically Active Euglobulin of Serum of a Donor Rabbit on the Activity of the Euglobulin of a Recipient Rabbit.—Experiments were designed to determine whether the active pyrogenic euglobulin of blood serum induced by pyrogens is in turn capable of converting the euglobulin of a recipient rabbit into a factor possessing pyrogenic activity. In other words, does the active euglobulin formed by bacterial pyrogens behave like pyrexin, or is it capable of transmitting its pyrogenic property to that particular plasma protein fraction in a recipient rabbit? The sera of rabbits were incubated in vitro for two hours with 0.1 mg. of the lipopolysaccharide of Westphal, and subsequently fractionated as previously described. In some rabbits the pyrogens (including the polysaccharide of Shear from *S. marcescens*) were injected intravenously and the blood was withdrawn 15 minutes to slightly over one hour afterwards. The collected serum was fractionated. All the plasma fractions were obtained and tested as previously described. Since the pyrogenic activity was located primarily in the euglobulin fraction, observations with only this fraction are herewith reported. The average results in 5 observations with the euglobulin fraction of the donor animals induced a rise of 2.5° F. This active euglobulin fraction resulting from the interaction of the pyrogen and the plasma was then injected in varying amounts in another recipient normal rabbit. The amounts injected ranged from 0.5 ml. to 5 ml. Wide variations in the amount of injected euglobulin were used to be certain that a sufficient quantity of the active euglobulin had been in-

jected in the recipient rabbit, and also to determine whether concentration of euglobulin had any influence on the ultimate effect. After an interval ranging from about one to over two hours, the recipient rabbit was bled. The serum was collected and accordingly fractionated. The euglobulin fraction was found to be wholly nonpyrogenic. The average rise in temperature in 13 experiments amounted to an increment of 0.1° F. In other words, the active euglobulin resulting from the original bacterial pyrogen injection is per se incapable of inducing an actively pyrogenic euglobulin in another rabbit. This effect is therefore similar to the behavior of pyrexin (Table III). The serum and plasma protein fractions (i.e., the pseudoglobulin-albumin and the alpha globulins) of the recipient rabbit were likewise inactive and failed to induce any fever in other rabbits.

The Effect of the Pyrogenic Euglobulin of Rabbits on the Temperature and Leukocyte Level of Dogs.—In earlier studies pyrexin was shown to induce in dogs an elevation in temperature, an initial leukopenia, and a subsequent leukocytosis.[20, 21] The leukopenia was observed to be referable to a leukopenic factor,[20] and the eventual developing leukocytosis to a thermostable leukocytosis factor.[21, 22] These two factors can be dissociated from crude pyrexin.[20, 22] If the converted euglobulin in the plasma of rabbit following the injection of either of the pyrogens studied is essentially a pyrexin-like substance, what effect would it have on the temperature and leukocyte level when injected intravascularly in dogs? The data of this series of experiments are assembled on Table V. The active insoluble euglobulin extracted from blood serum of a rabbit treated previously with one of the bacterial pyrogens, suspended in an aqueous medium, was injected intracardially in dogs, in concentrations of 0.8 ml. to 1 ml. In the majority of experiments, the euglobulin injected originated from in vitro incubation for one to two hours of the pyrogen and serum by the procedure already described. As indicated by the data on Table V, the injection of such converted rabbit euglobulin behaved precisely as crude pyrexin. There followed an elevation in temperature which within the first four hours averaged 3.3° F. There was an initial leukopenia; within the first two hours this averaged 2,183 leukocytes per cubic millimeter or a reduction from the base level of 81.4 per cent. Eventually a leukocytosis developed. The maximum level was, as a rule, reached on the following day yielding an average of 28,825 leukocytes per cubic millimeter or an increase of 145.2 per cent. The foregoing type of results indicates that the active euglobulin, formed by the interaction of the bacterial pyrogens utilized and of blood serum displays the same hematopoietic manifestations as crude pyrexin, in turn extracted from inflammatory exudates. Earlier studies have indicated that normal rabbit serum per se is nonpyrogenic in the dog.[2, 8]

Effect of Alpha Globulins from the Serum of a Rabbit Injected with Pyrogens on the White Blood Cell Level of Dogs.—The capacity of the converted euglobulin of rabbit serum following an injection of one of the bacterial pyrogens employed in this study, in inducing fever, leukopenia, and subsequent leukocytosis in dogs, has just been described (Table V). The question

which immediately arises concerns the specificity of these effects. Do other globulins from rabbit sera induce the same type of response in dogs? The alpha globulins were extracted after removal of the euglobulin fraction, as described. The additional presence of some small trace of admixed beta globulin appeared to be a possibility as suggested by preliminary paper electrophoretic studies, performed by Dr. J. Schulz. The electrophoretic studies indicated also the presence of at least traces of albumin in all of the "salted"

TABLE V. EFFECT ON THE TEMPERATURE AND ON THE LEUKOCYTE LEVEL OF DOGS CAUSED BY THE EUGLOBULIN OF BLOOD SERUM AFTER PYROGEN ADMINISTRATION

DOG NO.	EUGLOBULIN (PPT.) FROM SERUM OF RABBIT NO.	AMOUNT AND TYPE OF PYROGEN ADMINISTERED	METHOD OF ADMINISTRATION OF PYROGEN TO RABBIT SERUM	MAXIMUM ELEVATION IN TEMPERATURE ° F.	BASAL LEVEL OF LEUKOCYTES PER C.MM.	MINIMUM LEVEL OF LEUKOCYTES PER C.MM.	MAXIMUM LEVEL OF LEUKOCYTES ON FOLLOWING DAY PER C.MM.
334-T	22-60	4 mg. (S.)*	Intravenously 20 min. before bleeding	1.9	11,550	1,550	42,000
257-T	22-59	±2-3 mg. (S.)	Intravenously 3 hr. and 11 min. before bleeding	2.5	12,900	2,950	31,400
262-T	22-61	7.5 ml. serum + 0.7 mg. (S.)	In vitro incubation for 2 hr.	2.9	11,850	2,450	32,850
336-T	22-71	10 ml. serum + 0.7 mg. (S.)	In vitro incubation for 2 hr.	4.4	13,000	3,600	22,750
339-T	22-78	14 ml. serum + 0.1 mg. (L.p.)†	In vitro incubation for 2 hr.	3.8	9,775	1,150	20,750 (6 hr. later)‡
340-T	22-78	14 ml. serum + 0.1 mg. (L.p.)	In vitro incubation for 2 hr.	4.2	11,450	1,400	23,200 (4 hr. later)‡
Average	-	-	-	3.3	11,754	2,183	28,825

*(S.) refers to Shear's polysaccharide from *S. marcescens.*
†(L.p.) refers to Westphal's lipopolysaccharide from *B. coli.*
‡On the following day the WBC were as follows: 339-T = 23,550; 340T = 34,600.

out fractions utilized in this study. However, when the albumin fraction was studied following the removal of the gamma, alpha, and beta globulins, no pyrogenic activity could be elicited in the injected animals. The active fever-inducing euglobulin of rabbit blood serum was confirmed by paper electrophoresis to be primarily a gamma globulin.

The "globulins" (primarily alpha globulins) were found, except in one case, to be nonpyrogenic. The average of 5 experiments yielded an increase of 0.6° F. (Table VI). About 2 ml. of these globulins in an aqueous solution were injected intracardially in dogs. The peripheral white blood cell count

was measured every hour for a period of about 6 hours. On the following morning, observations on the leukocytic counts were continued for 2 or 3 hours. The data assembled in Table VI indicate that with one exception the injections of these globulins induced no significant leukopenia. There was an average drop in the number of circulating leukocytes from 11,295 to 9,530 or a decrease of 15.6 per cent. Contrast this with a decrease of 81.4 per cent

TABLE VI. EFFECT ON THE TEMPERATURE AND THE LEUKOCYTE LEVEL OF DOGS CAUSED BY THE "GLOBULINS" OF BLOOD SERUM AFTER PYROGEN ADMINISTRATION*

DOG NO.	"GLOBULINS" FROM SERUM OF RABBIT NO.	AMOUNT AND TYPE OF PYROGEN ADMINISTERED TO RABBIT	METHOD OF ADMINISTRATION OF PYROGEN TO BLOOD SERUM	MAXIMUM ELEVATION IN TEMPERATURE ° F.	BASAL LEVEL OF LEUKOCYTES PER C.MM.	MINIMUM LEVEL OF LEUKOCYTES WITHIN 1-2 HR. PER C.MM.	MAXIMUM LEVEL OF LEUKOCYTES (2-6 HR. DETERMINATION) PER C.MM.	LEUKOCYTE LEVEL ON FOLLOWING DAY PER C.MM.
337-T	22-59	±2-3 mg. (S.)†	Intravenously 3 hr. and 11 min. before bleeding	0.0	9,225	11,000	11,850 (2 hr. later)	8,100
338-T	22-71	10 ml. serum + 0.7 mg. (S.)	In vitro incubation for 2 hr.	1.1	10,775	3,500	11,600 (6 hr. later)	11,050
341-T	22-78	14 ml. serum + 0.1 mg. (L.p.)‡	In vitro incubation for 2 hr.	-0.1	11,325	8,550	13,250 (5 hr. later)	12,400
342-T	22-78	14 ml. serum + 0.1 mg. (L.p.)‡	In vitro incubation for 2 hr.	0.0	10,525	11,550	17,550 (4 hr. later) 11,300 (5 hr. and 28 min. later)	-
344-T	22-77	2.3 mg. (L.p.)	Intravenously 1 hr. and 6 min. before bleeding	1.8	14,625	13,050	20,100 (5 hr. later)	17,550
Average				0.6	11,295	9,530	14,275	12,275

*The "globulins" administered refers primarily to alpha globulins probably admixed with some beta globulins.

†(S.) refers to Shear's polysaccharide.

‡(L.p.) refers to Westphal's lipopolysaccharide.

when the euglobulin fraction was utilized (cf. Table V). The subsequent rise in the number of circulating leukocytes was also found to be quite insignificant, averaging 26.4 per cent within 6 hours, and 8.6 per cent on the following day. This stands in sharp contrast compared with the average rise of 145.2 per cent following the injection of the active euglobulin fraction (Table V).

In conclusion, the effect in dogs of the euglobulin of rabbit serum obtained, following the intravenous injection of either of the bacterial pyrogens

studied is quite specific. It induces an augmentation in temperature, an initial leukopenia, and a subsequent leukocytosis (Table V). With the use of mainly the alpha globulins these effects are essentially absent (Table VI).

The Effect of Lower Concentrations of a Pyrogen and of "Piromen" on the Formation of a Pyrogenic Euglobulin.—Pyrexin can possibly be envisaged as an end product, possibly of necrosin hydrolysis. Necrosin is a toxic euglobulin, recovered primarily from acid exudates, which seems to be responsible to a large extent for the pattern of injury with inflammation.[24] Closely associated with it is a pyrogenic factor, termed pyrexin.[2] Pyrexin can be dissociated from necrosin and it can be crystallized.[2, 3] Necrosin has been shown to contain proteolytic activity.[27] Incubation of nonpyrogenic necrosin frequently induces the formation of a pyrogenic factor which is probably identical to pyrexin.[2] For these reasons pyrexin can possibly be considered as the end product of enzymatic hydrolysis by necrosin. The injection of pyrexin induces an elevation in temperature, and the fever centers in the hypothalamic region are apparently affected.[24, p. 113, 29] It is conceivable that the active pyrogenic euglobulin formed by the interaction of a bacterial pyrogen and plasma is pyrexin-like in nature. If that were so, one might envisage that with the large dose of lipopolysaccharide employed (0.6 to 3 mg.), there may be a surplus of euglobulin formed in the circulation. As a consequence, upon bleeding the animal, there would be an excess of the actively converted euglobulin which in turn could be recovered, and like pyrexin would induce an elevation in temperature when injected into rabbits. If this view of a surplus formation of euglobulin with large doses of the pyrogen were correct, it should follow that with much smaller amounts (one-tenth or less) of the same bacterial pyrogen there should develop, as in the case of pyrexin, only an elevation in temperature without the additional formation of any active pyrogenic euglobulin. The observations in Table VII support this view.

TABLE VII. THE BACTERIAL EFFECT OF LOW DOSES OF A BACTERIAL PYROGEN ON THE CAPACITY OF THE EUGLOBULIN TO INDUCE FEVER IN RECIPIENT ANIMALS

DOSE OF LIPOPOLYSACCHARIDE UTILIZED IN RABBIT (GAMMA)	MAXIMUM RISE IN TEMPERATURE INDUCED IN RABBIT PRIOR TO WITHDRAWAL OF BLOOD BY INJECTING LOW DOSE OF LIPOPOLYSACCHARIDE ° F.	MAXIMUM RISE IN TEMPERATURE INDUCED IN RECIPIENT RABBITS BY EUGLOBULIN DERIVED AFTER ADMINISTRATION OF LOW DOSE OF LIPOPOLYSACCHARIDE IN A DONOR RABBIT ° F.
50	1	0.8
		0.4
70	1.5	0.2
		0.4
Average	1.25	0.45

It is therefore evident that in contrast with the much larger doses of lipopolysaccharide (i.e., 0.6 to 3 mg.), low doses of this pyrogen (50-70γ), as encountered in the pyrexin-treated rabbits (Table III), fail to form an active pyrogenic euglobulin. The other plasma protein fractions (i.e., pseudoglobulin-albumin, or mainly the alpha globulins) with low doses of pyrogen are

likewise inactive. In view of these facts it appears quite possible that the formed euglobulin resembles pyrexin in its biological activity.* Both pyrexin and the formed euglobulin seem to be end products capable of elevating the temperature of injected rabbits. The significant point appears to consist in the fact that a high concentration of the bacterial pyrogen forms a surplus of the pyrogenic euglobulin which in turn can elicit appreciable fever in rabbits, whereas the administration of low doses of the same pyrogen fails to produce a surplus of the active euglobulin or pyrexin-like material. To recapitulate, pyrexin and the formed euglobulin following the injection of the bacterial pyrogens utilized in this study, resemble each other in the following properties:

1. Both pyrexin and the formed euglobulin of serum are either euglobulins or components from the respective euglobulin fraction of exudate or of blood plasma.

2. Both induce in dogs fever, initial leukopenia, and subsequent leukocytosis.

3. Both seem to be end products. Though they induce fever and the afore-mentioned hematological effects, each of these two factors is incapable of forming an active pyrogenic euglobulin when injected into a recipient rabbit.

Some studies were also undertaken with the commercial product Piromen. It is quite possible that this substance may likewise be an end product. Irrespective of concentration (i.e., 10 to 6,000 gamma) Piromen was incapable of converting the euglobulin of rabbit serum into a pyrogenic factor. This material failed to give the usual tests for proteins and appears to contain polysaccharides. However, a precursor of the finished product was obtained in the form of a crude Piromen powder through the kindness of Dr. H. Feevold of the Baxtor Laboratories. This crude material had many characteristics reminiscent of pyrexin. In varying concentrations ranging from 3 to 22 mg. Piromen powder also failed after intravenous injection to convert the euglobulin fraction of plasma into a pyrogenic factor. This material, like crude pyrexin, elicited a positive Biuret and Molisch test for the presence of nitrogenous and carbohydrate groups. Piromen powder resembled pyrexin in the amino-nitrogen determined before and after hydrolysis. Paper chromatographic studies by Dr. W. Kalnins indicated that crude pyrexin and crude Piromen powder yielded precisely the same configuration. Each had 16 similar spots on the chromatogram. These facts suggested a close resemblance of these two pyrogenic substances, i.e., one derived from exudates, and the other extracted from Pseudomonas bacilli. Smith and associates[28] stated in their textbook that Pseudomonas contain a protease. It is therefore conceivable that the crude Piromen material and pyrexin are both protein breakdown products, the Piromen powder being a product of bacterial proteolysis and py-

*Pyrexin induces an inactive serum; while low doses of the pyrogenic bacterial lipopolysaccharide induce a pyrogenic blood serum. This is the only difference in the analogy. Westphal has pointed out that the lipopolysaccharide is effective in quantities as small as 0.001-0.002 μg kg. (given intravenously).[17] It is therefore quite possible that the injection of 50 to 70 units of lipopolysaccharide in rabbit (Table VII) still leaves a sufficient trace of this unchanged and potent pyrogen in the circulation. This would render the whole blood serum pyrogenic; but still there is an insufficient quantity of the pyrogen to form a surplus of the actively pyrogenic euglobulin.

rexin being a proteolytic product of injured cells in exudates. This close similarity to pyrexin would perhaps reasonably explain the incapacity of Piromen as an end product to convert the euglobulin of rabbit plasma into a pyrogenic factor.*

DISCUSSION

This study indicates that the two bacterial pyrogens studied, when injected intravenously into rabbits, seem to convert the euglobulin of blood plasma into an active pyrogenic substance. This active fever-inducing euglobulin seems to behave in a manner similar to pyrexin, the pyrogenic factor recovered primarily from acid exudates.[2] It is not certain whether the bacterial pyrogen actually converts the euglobulin of the plasma into a pyrogenic euglobulin, or whether it merely fixes itself on this protein fraction forming thus a pyrogenic-globulin complex. The present observations indicate that the bacterial pyrogens studied alter selectively the euglobulin fraction, conferring upon the latter definite pyrogenic properties. This pyrogenic euglobulin has the same properties as pyrexin extracted from inflammatory exudates. Since pyrexin is also located in the euglobulin fraction of inflammatory exudates, it appears that the bacterial pyrogens have altered the euglobulin of plasma into a pyrexin-like substance. The endogenous pyrogen postulated by Grant[11] and Whalen after the injection of a bacterial pyrogen may be identical or similar to the active pyrogenic euglobulin described in the present communication. The facts reported can reasonably explain the curious observation that bacterial pyrogens and pyrexin have similar biological properties following their respective injections into animals. Both are capable of eliciting fever, initial leukopenia, and subsequent leukocytosis.

A comparison of the in vivo and in vitro observations reported in this communication brings to light an interesting consideration. The lack of any specific localization by in vitro studies in the protein fractions of serum in contrast to the in vivo formation of the euglobulin fractions suggests the participation of either viable cells or an enzyme in the intact circulation favoring the formation of a specific pyrogenic euglobulin. The stability of this formed product is at present under study.

Finally, the formation by bacterial pyrogens of a pyrogenic euglobulin in the blood stream that appears to be pyrexin-like in nature may prove a convenient procedure for preparing this pyrogenic substance in abundance for possible clinical application. It would appear to be of interest to determine also whether the pyrogenic euglobulin can be dissociated from its leukopenic and leukocytosis-inducing properties, as has been accomplished in the case of pyrexin.[20, 21, 22] Such studies are now in progress.

SUMMARY AND CONCLUSIONS

1. Pyrexin, the pyrogenic factor of primarily acid exudates, in concentrations such as are recovered from inflammatory exudates, fails to induce any

*The blood serum after administration of Piromen powder, as in the case of pyrexin, was found pyrogenically inactive.

tolerance following repeated injections of this substance into rabbits. Tolerance to low doses of pyrexin (0.25 mg.) is probably not of great importance, since pyrexin is found in higher concentration in the exudate (1.2-9.8 mg.). The biological significance of pyrexin from the standpoint of tolerance resides primarily in the concentration of this substance in exudative material. The inability to induce tolerance in concentrations such as are found in exudates renders the view quite plausible that pyrexin is an important factor in the mechanism of fever with inflammation. It is admitted, however, that there may perhaps be other additional fever-inducing factors present in an inflamed area, particularly in alkaline exudates.[6]

2. The view that pyrexin is contaminated by bacterial pyrogens during the extractive procedure is a remote possibility for the following reasons: (a) In contrast to other chemical factors derived also from exudates, pyrexin is biologically specific in inducing fever. This holds even though the *initial* extractive procedure for some of these factors is identical to that of pyrexin. (b) Normal blood serum extracted for pyrexin by a similar technique does not induce any elevation in temperature. (c) Pyrexin extracted from alkaline inflammatory exudate is usually inactive in contrast to its activity when derived from acid exudates.

3. The injection into the circulating blood of two different bacterial pyrogens, namely, the polysaccharide of Shear from *Serratia marcescens,* and the lipopolysaccharide of Westphal from *E. coli,* favors the transformation of the euglobulin of rabbit blood plasma into a markedly pyrogenic factor. In comparison to the euglobulin the other protein fractions of plasma remain definitely less pyrogenic. It is not established whether the pyrogenic euglobulin formed by bacterial pyrogens is a true conversion or merely a bacterial pyrogen-euglobulin complex formed in the blood stream.

4. The formation of the pyrogenic euglobulin is an in vivo phenomenon. In vitro incubation of blood serum with a bacterial pyrogen apparently fails to localize the effect primarily in the euglobulin fraction. The use of markedly different amounts of the pyrogens indicates that the in vitro effect is probably not referable to excessive concentration of the pyrogen.

5. The active pyrogenic euglobulin induced marked fever, an initial leukopenia, and a subsequent leukocytosis in dogs. These effects on temperature and on the hematopoietic picture are similar to the effect elicited by crude pyrexin on that animal.

6. Very low concentrations of one of the bacterial pyrogens studied (lipopolysaccharide from *E. coli*) failed to produce, in contrast to the higher concentrations of that pyrogen, an active euglobulin which in turn induced fever in a normal rabbit. The effect is reminiscent of the effect elicited by pyrexin. The latter also failed to form a pyrogenic euglobulin.

7. It is concluded that pyrexin and the actively formed euglobulins are end products capable of inducing fever, initial leukopenia, and subsequent leukocytosis. A surplus of the active euglobulin formed by large concentrations of the bacterial pyrogens reasonably explains the transmission of the

pyrogenic effect by injection of the formed euglobulin fraction. On the other hand, the recipient of such a pyrogenic euglobulin fails to form an active euglobulin which would be pyrogenic to another rabbit. This effect is similar to the one induced by pyrexin.

8. Pyrexin from inflammatory exudates and the formed pyrogenic euglobulin caused by the interaction in the circulating blood of some bacterial pyrogens seem essentially similar in nature. The exception manifested by commercial Piromen is discussed, and a possible interpretation is presented for this observation.

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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA COMPLICATED BY ACQUIRED HEMOLYTIC ANEMIA TREATED WITH CORTISONE

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IT IS well established that the defect in paroxysmal nocturnal hemoglobinuria is in the red cell itself. Recently we had an opportunity to observe four new cases of this disease.[1] In one of these cases a secondary hemolytic defect residing in the plasma was observed, apparently due to autoimmunization. Cortisone had a very beneficial effect in this one case, as it has in most cases of acquired hemolytic anemia. In contrast to this the results of steroid therapy have been variable in uncomplicated paroxysmal nocturnal hemoglobinuria. In fact, Crosby[2] has reported that cortisone is probably contraindicated in paroxysmal nocturnal hemoglobinuria because of its tendency to cause spontaneous thrombosis. This tendency is already present in paroxysmal nocturnal hemoglobinuria and is probably the most serious threat to life in that disease. We wish to report this case in more detail with particular reference to studies on the formation of antibodies against all blood cellular elements and the results of treatment with cortisone.

METHODS

1. Serum hemoglobin was done by the method of Hunter.[3]

2. Urine hemoglobin was measured using a modification developed in this laboratory whereby hemoglobin is first precipitated from solution, and the redissolved precipitate is measured as alkali hemochromogen. The method is as follows: To 100 ml. aliquot of urine is added 70 Gm. of ammonium sulfate. After standing for two hours, the precipitated protein is collected by filtration. The precipitate is dissolved in 100 ml. of 10 per cent sodium hydroxide, and the hemoglobin is then estimated by the hemochromogen method of Hunter.[3]

CASE REPORT

A 41-year-old Filipino man was well until 1944, when he was hospitalized at the 51st General Hospital in New Guinea because of easy fatigability and multiple ecchymoses and petechiae. His red cell count was 1.3 million c.mm., white cells were 3,500 c.mm. with 36 per cent neutrophils and 64 per cent lymphocytes. The platelet count was 185,000 per cubic millimeter. His bone marrow was acellular, and because of this a diagnosis was made of aplastic anemia possibly due to Atabrine. He was treated with whole blood transfusions. In March, 1954, he was transferred to the Birmingham General Hospital in Van Nuys, California, where the findings were essentially the same, except for the presence of a thrombocytopenia, the lowest of which was 15,000 per cubic millimeter. From 1945 to 1949, he was apparently well. In 1949, the patient began to require three to four pints of whole blood every three months because of recurring anemia. These episodes of

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anemia were always heralded by scleral icterus and darkening of the urine, the latter being noted especially in the morning. In 1953, he began to experience reactions after each transfusion which consisted of shaking chills followed by "red urine" in the morning.

The past history was negative. He was a gardener with no known exposure to chemical toxins. His father, mother, six siblings, and four children were living and well.

He was first admitted to this hospital on Jan. 9, 1954, because of anemia. He was a well-developed, well-nourished, but obviously icteric man. His liver was 3 cm. below the costal margin in the midclavicular line; the spleen was nontender and firm, and projected 3 cm. below the costal margin. The urine was negative for bile. The Bromsulphalein retention, cholesterol, cholesterol esters, prothrombin time, thymol turbidity, and alkaline phosphatase were within normal limits. There were 13.4 mg. of urobilinogen excreted in the urine in a 24-hour period. The serum bilirubin was 0.22 mg. per cent direct, and 2.9 mg. per cent indirect. Hemoglobin: 9.6 Gm. per cent, red cell count, 3.0 million per cubic millimeter. White cells: 4,300 per cubic millimeter with 50 per cent neutrophils, 35 per cent lymphocytes, 3 per cent monocytes, 1 per cent eosinophils, and 1 per cent basophils. Platelet count: 146,000 per cubic millimeter. The red cell fragility demonstrated hemolysis beginning at 0.48 per cent of saline and complete at 0.26, with a control of 0.48 to 0.36. A sickling preparation was negative. The bone marrow demonstrated marked erythrocytic hyperplasia and a slight increase in the number of "mast" cells. The direct Coombs test done with commercial antiserum was negative. However, a repeat test by Dr. Rose Payne at Stanford Medical School showed weak reactions in dilutions up to 1:80. There was no evidence of autoagglutination on incubation at 37° C. Cold agglutinins were positive to 1:64. The Donath-Landsteiner test was negative. The Ham test showed marked hemolysis of the patient's cells in normal serum, but also slight hemolysis of normal cells in the patient's serum. Further tests showed that this hemolysin was active against red cells of all blood types. The patient's morning plasma contained 129 mg. of free hemoglobin per 100 ml., while in the afternoon there were 92 mg. The serum from the same morning specimen contained 226 mg. per 100 ml. when allowed to clot at 37° C. for one hour. The Crosby test was strongly positive (the free hemoglobin level in the tube with thrombin was 200 mg. per 100 ml.; the control tube had 99 mg.).

The diagnosis of paroxysmal nocturnal hemoglobinuria having been confirmed, the patient was started on Dicumarol, maintaining a prothrombin time calculated by the Link-Shapiro modification of the Quick method of approximately 30 per cent. Following this he stated that his urine was free of blood for the first time. He was given four transfusions of washed red cells, but had repeated and severe transfusion reactions with chills and fever followed by increased amounts of hemoglobin in the urine the next morning. The transfusions temporarily raised his hemoglobin level to 10 Gm., but within ten days it was back to the original level of 8 Gm. The patient maintained this level on Dicumarol therapy, and was discharged on Feb. 11, 1954. He complained that he felt only moderately well following discharge and was able to work only intermittently because of recurring episodes of weakness. (The prothrombin times fluctuated between 30 and 60 per cent of normal as performed under the auspices of his local physician.)

His second admission was on May 10, 1954. One week prior to this entry he awakened with a temperature of 103° F. The following morning his urine was "very bloody." Since that time he had been quite weak. Physical examination was unchanged, except that the spleen and liver were now 5 cm. below the costal margins. The hemoglobin was 8 Gm. Red cell count: 2.8 million per cubic millimeter. White cells: 2,350 per cubic millimeter. Platelets: 246,000 per cubic millimeter. The indirect bilirubin was again elevated. The Coombs test was negative in all dilutions. The Ham test was again strongly positive. The 24-hour urinary hemoglobin excretion was 332 mg. Addition of thrombin (the Crosby test) again markedly increased the hemolysis. The patient was given two units of washed red cells following which the hemoglobin rose to 11 Gm. He was given a single intramuscular injection of 25 units of adrenocorticotropic hormone prior

to the transfusion in an attempt to avoid his usual hemolytic reaction. Dicumarol was given in sufficient dose to keep his prothrombin time below 30 per cent of normal, and his hemoglobin remained at 11 Gm.

The third hospital admission was on Aug. 9, 1954. Physical examination was again unchanged. The red blood cell count was 2.3 million per cubic millimeter with 6.0 Gm. of hemoglobin. The white count was 2,000 per cubic millimeter with 61 per cent neutrophils, 34 per cent lymphocyptes, 3 per cent monocytes, 1 per cent eosinophils, and 1 per cent basophils. The platelet count was 136,000 per cubic millimeter. The cold agglutinins were negative in all dilutions. The previously noted serum hemolysin was again found and appeared to be stronger than before, although it was not quantitated. The patient's serum also contained agglutinins against white cells. (This test was done by Dr. Rose Payne at Stanford Medical School.) For the first time the direct Coombs test was positive with commercial antiserum to a dilution of 1:160.

The dose of Dicumarol was increased, thus lowering his prothrombin time to an effective therapeutic range. He was then given two units of washed red cells which raised the hemoglobin to 9.6 Gm. per cent and the red blood cell count to 2.8 million per cubic millimeter. After consideration of the fact that Dicumarol plus transfusions seemed ineffective in the control of his disease, and also in view of the hemolysin in the patient's serum, he was started on cortisone with an initial dose of 25 mg. orally four times daily.

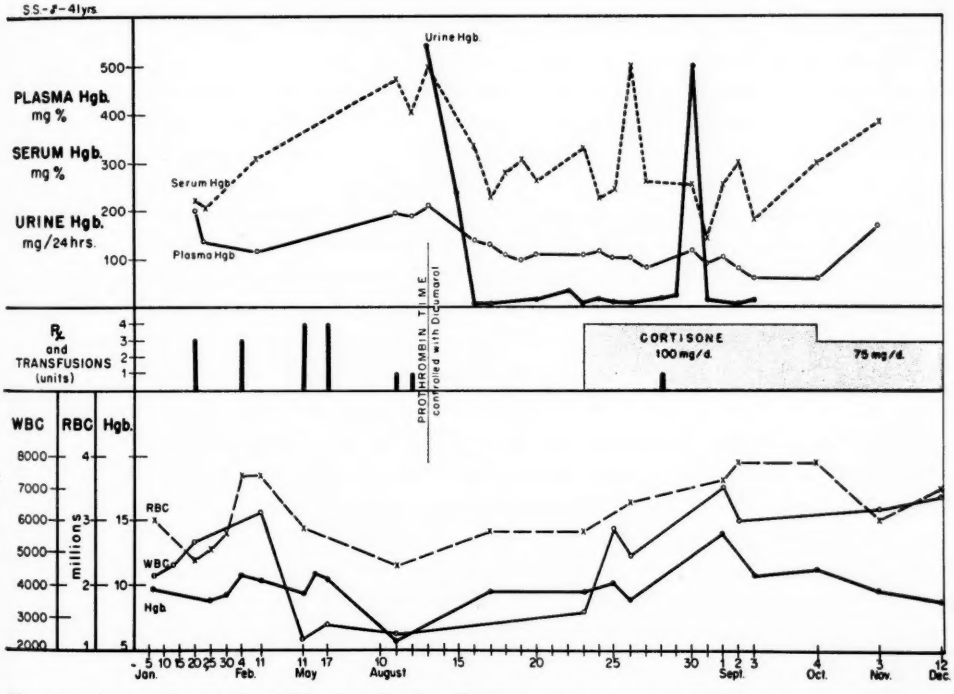

Fig. 1.—Composite graph of blood and urine findings prior to and following cortisone therapy. Dicumarol was given throughout with prothrombin times controlled.

The patient's course may be followed in Fig. 1 which gives details of the blood counts, the 24-hour urinary hemoglobin determinations, and the plasma and serum hemoglobins. Serum hemoglobins were determined after incubation at 37° C. for one hour; the difference between these and the plasma hemoglobin is believed to reflect the activity of the paroxysmal nocturnal hemoglobinuria hemolytic system. The plasma hemoglobin, on the other hand, should be the result of both paroxysmal nocturnal hemoglobinuria activity and the acquired hemolysin. He was given one unit of washed red cells on July

28, 1954, with some darkening of the urine the following day, but without a clinical reaction. The hemoglobin rose to a maximum of 13.9 Gm. per cent falling slowly to 11.1 Gm. per cent by Sept. 2, 1954 (Fig. 1). To us this seemed of significance considering the small rises with previous transfusions. Following the administration of cortisone, the white count rose from markedly low levels to what would be considered normal limits with the neutrophilic series showing the greatest increase. The differential count was 77 per cent neutrophils, 17 per cent lymphocytes, 1 per cent monocytes, and 1 per cent eosinophils.

During the succeeding month as an outpatient the prothrombin time was adequately controlled with Dicumarol, and the cortisone was continued at a dose of 100 mg. per day. The patient felt exceedingly well and was able to work full time. As noted on the chart the hemoglobin and total red count were maintained at the previous level. The Coombs test was positive 1:40 on Oct. 4, 1954. At this time the dosage of cortisone was reduced to 75 mg. per day because of the appearance of "moon" facies. He was seen again on Nov. 3, 1954, and as noted on the graph, he had maintained a normal total white count and differential. The total red cell count and hemoglobin had decreased slightly (Fig. 1) but he continued to feel well and was working full time. He was again seen on Dec. 7, 1954. The peripheral blood findings were essentially unchanged. The patient appeared to have stabilized at this level of hemoglobin. Clinically, he appeared well, and stated that he felt strong and had no symptoms. There was a weight gain of 12 pounds and while he was not edematous, there was probably some dilution factor present accounting for the discrepancy between the hemoglobin level and the state of well-being. Since he seemed to have adjusted to this level of hemoglobin, no attempt was made to increase the dose of cortisone to the original level of 100 mg. per day.

He has now been observed for a total of eight months, and at present is feeling very well with a stable level of hemoglobin.

DISCUSSION

Paroxysmal nocturnal hemoglobinuria is presently considered to be the result of a defect in the protein martix of the red cell. In the typical case, there is no hemolytic effect demonstrable in the plasma, as can be proved by the fact that normal red cells transfused into patients with paroxysmal nocturnal hemoglobinuria have a normal survival time. On the other hand, evidences of antibody formation against blood cells have been reported in these cases at a frequency far above that which might be expected in a random population. Crosby reviewed the literature in 1953,[2] and found three cases with serum hemolysins active against normal red blood cells, and two more in which there were serum hemolysins active against red cells of the patient's own blood group. One of the latter had a positive Coombs test during a crisis. There were six other cases which were reported to have autoagglutinins, and two which had cold agglutinins. In addition to the cases quoted by Crosby, there is a report by Hollander[4] of a case with a positive Coombs test, and one by Ciocco of a patient whose serum hemolyzed normal red blood cells.

The first indication of a complication in our patient came from a transfusion reaction that occurred despite the fact that compatible red cells had been washed nine times in saline prior to administration. Washed red cells were given because of Crosby's work[5] which demonstrated that the frequent transfusion reactions in paroxysmal nocturnal hemoglobinuria patients may be due to a factor in the normal donor plasma. According to Crosby, such reactions can be prevented by using red cells washed three to four times with normal saline. In retrospect, the reaction we encountered in our patient was

probably due to hemolysis of the *donor* cells by a hemolysin in the patient's plasma. The second evidence of a complication was detected in the control tube of the Ham test, where it was noted that the patient's serum caused slight hemolysis of normal compatible red cells. At this time, the direct Coombs test on the patient's red cells was negative using commercial Coombs serum, but a weak positive reaction was obtained by Dr. Rose Payne at Stanford Medical School using serum she had prepared herself. At the time of his third admission, the Coombs test was strongly positive, and a white cell agglutinin was also present.

Since cortisone is generally agreed to be the treatment of choice in acquired hemolytic anemia, we administered it to our patient. Cortisone and adrenocorticotropic hormone have been tried on presumably uncomplicated cases of paroxysmal nocturnal hemoglobinuria with variable results. Martin[6] reported the case of a 36-year-old man in which the only beneficial treatment was 100 mg. of adrenocorticotropic hormone daily, with a complete remission for eighteen months. Martin made no mention of the Coombs test. Fudenberg's[7] case was treated with 40 units of adrenocorticotropic hormone daily, resulting in a remission that had lasted six months at the time of writing. The Coombs test was negative. Kalant and Cyr[8] treated a 41-year-old man for four years, administering adrenocorticotropic hormone on three occasions. They obtained a remission only on the second trial, though short-lived. There were no abnormal antibodies. Hoffman and Powell[9] had one case that did not demonstrate abnormal antibodies, and did not respond to adrenocorticotropic hormone. Hollander's[4] case with a positive Coombs test improved with administration of adrenocorticotropic hormone. Crosby[2] reported six cases in which adrenocorticotropic hormone was used. He concluded that it was without benefit, and, in fact, was contraindicated because of the increased thrombotic tendency associated with its use. Further, he quoted two instances of death during adrenocorticotropic hormone therapy from coronary thrombosis and portal vein thrombosis.

Since our patient had demonstrated some improvement with Dicumarol therapy, and in view of the alleged effect of cortisone in producing thrombosis, he was maintained on Dicumarol when cortisone treatment was begun. The results of cortisone therapy were very favorable. His hemoglobin rose to a high level following two units of washed red cells, whereas previously he had shown very little response to transfusions other than increased hemolysis. The plasma hemoglobin level and the urine hemoglobin output were considerably reduced. The serum hemoglobin level remained unchanged, indicating that the beneficial effect of cortisone was not exerted on the plasma factors of the paroxysmal nocturnal hemoglobinuria hemolytic system. This, however, does not rule out the possibility of a direct protective effect on the abnormal red cells of paroxysmal nocturnal hemoglobinuria. In the succeeding four months no further transfusions were required, although prior to this, he had required three to four units of blood every three months. The patient was subjectively well for the first time; he looked better clinically than his hemoglobin and red cell count would indicate.

The high incidence of autoantibodies in paroxysmal nocturnal hemoglobinuria is somewhat surprising. We are inclined to agree with Crosby that their presence is a complication, and is not related to the basic pathologic physiology of paroxysmal nocturnal hemoglobinuria. The possibility of many transfusions playing a role in the production of these antibodies is raised, but the incidence of autoimmunization is far less in aplastic anemia patients who also usually receive large numbers of blood transfusions. It would therefore seem that paroxysmal nocturnal hemoglobinuria falls in the same category as chronic lymphocytic leukemia and disseminated lupus erythematosis, both of which diseases show a higher incidence of autoimmunization. Dausset[10] has reported the occurrence of white cell agglutinins in a number of cases of paroxysmal nocturnal hemoglobinuria. Our case adds another to this group. While Crosby[2] has demonstrated that leukopenia and thrombocytopenia in paroxysmal nocturnal hemoglobinuria are probably due to the same factors that cause red cell hemolysis, antibodies may be partially responsible in some cases. In our case the rise in the white count following administration of cortisone suggests that the white cell agglutinin may have been the chief cause of the leukopenia.

A careful survey for red cell antibodies in cases of paroxysmal nocturnal hemoglobinuria, using all possible techniques would probably uncover a far higher incidence of this complication than has been reported. Demonstration of an antibody effect in vitro does not necessarily indicate that it is of clinical significance. Direct hemolysis of normal cells in a test tube or shortened survival time of transfused normal red cells would be proof that the antibody exerted an effect. On the other hand, Dacie[11] has demonstrated that the red cells of paroxysmal nocturnal hemoglobinuria are unusually susceptible to hemolysis by antibodies that may ordinarily only agglutinate normal red cells. This tendency for the cells of paroxysmal nocturnal hemoglobinuria to be hemolyzed by a wide variety of immune antibodies makes the development of autoimmunization unusually serious. Fluctuation in the titer of these antibodies may explain some of the variation in severity of the disease which has been noted.

The beneficial effect of cortisone in our case is similar to that observed in the treatment of a majority of cases of acquired hemolytic anemia. In view of Crosby's warning regarding the possibility of thrombotic tendencies when cortisone is administered, it is probably wise to maintain patients with paroxysmal nocturnal hemoglobinuria on Dicumarol when administering the steroid hormones. Our experience with this patient suggests that if a case of paroxysmal nocturnal hemoglobinuria is not doing well, an exhaustive search for red cell antibodies is indicated. If evidence of autoimmunization is found, cortisone should be tried. It is possible that in the previously reported cases some of the variation noted in response to adrenocorticotropic hormone was the result of the presence of hemolysins in some, and that only those with antibodies can be expected to respond well. Some of the occasional beneficial results following splenectomy may be similarly explained.

SUMMARY

1. A case of paroxysmal nocturnal hemoglobinuria, complicated by the presence of a serum hemolysin active against normal red cells is reported.

2. A review of the literature points out that such autoimmunization is surprisingly frequent in this disease. The reason for this is obscure.

3. Cortisone successfully controlled the complicating acquired hemolytic anemia in this case.

4. A careful search for red cell antibodies should be made in cases of paroxysmal nocturnal hemoglobinuria that are not responding well to Dicumarol treatment. If autoimmunization is present, cortisone is the treatment of choice. Dicumarol should be administered concurrently to suppress the hemolytic process of paroxysmal nocturnal hemoglobinuria, and to minimize the alleged thrombotic effect of cortisone.

We wish to thank Mrs. Jane Fenton and Harold T. Wrenn for carrying out the various laboratory procedures on this patient.

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A STUDY OF TWO INTRAVENOUS FAT EMULSIONS IN HUMAN SUBJECTS*

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INTRODUCTION

THE principal side-reaction attending the intravenous infusion of fat emulsions is fever. The mechanism of its production is unknown.[1] Although intravenous infusion of fat emulsions has been studied on an experimental basis in hundreds of human subjects during the past several decades, few attempts have been made to correlate biochemical and hematological changes during and following the infusions with the febrile response. The present study represents such an attempt, with the goal of finding clues to the genesis of the temperature elevation.

The concentration of fat emulsion in blood serum was followed by measurements of serum optical density and serum total fatty acid concentration. The free fatty acid concentration of serum was also measured because of the interest of this laboratory in this lipid moiety and its possible participation in fat metabolism.[2]

Bennett and Beeson[3] have demonstrated that polymorphonuclear leukocytes contain a substance which produces fever when injected intravenously in rabbits. This substance could not be extracted from any of the many other tissues studied by them. It has been suggested[4] that release of endogenous pyrogen from inflammatory cells may be involved in the fever of human disease. Kerby[5] has shown that injured or lysed leukocytes release lysozyme into the surrounding medium. For these reasons white blood cell counts and serum lysozyme determinations were performed in order to examine the possibility that the febrile response to fat emulsions involves this mechanism.

METHODS AND PROCEDURES

The emulsions used in this study were supplied by the Department of Nutrition of the Harvard School of Public Health and by the Upjohn Company, Kalamazoo, Michigan.† The composition of the emulsion from the Harvard School of Public Health, prepared June 9, 1954, serial No. HN 58E, is as

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follows: refined cottonseed oil (OSG No. 2 SRRL) 15 per cent; purified soybean phosphatides 1.2 per cent; polyethoxy propylene oxide (Pluronic F-68) 0.3 per cent; dextrose (anhydrous) 5.0 per cent. The composition of the Upjohn emulsion, prepared Nov. 17, 1953, and designated as Lipomul Res. No. 10, 224-8, is as follows: olive oil 15 per cent; dextrose 4.2 per cent; soybean phosphatide 1 per cent and polyethoxy propylene oxide (Pluronic F-68) 0.5 per cent. These emulsions are referred to as HSPH and Lipomul, respectively, in the remainder of this report.

Nine normal men who were fasting received one 500 ml. intravenous infusion of each emulsion one week apart. The order of administration of the two emulsions was random.

Before the infusions were started, a sample from each bottle was examined for particle size by dark-field microscopy. Almost all particles were under 1 micron in diameter, with no particles larger than 3 microns.

The intravenous infusion was started with 5 per cent dextrose in water. The fat emulsion was run into the tubing of the dextrose infusion. The dextrose infusion was stopped when the fat emulsion infusion was begun. The fat emulsion was given at 30 drops per minute for the first hour and 60 drops per minute for the remaining period. The average time required for the infusion was 3.6 hours.

Blood pressure, respiration rate, pulse rate, and rectal temperatures were recorded before and at intervals during and after the infusions. Clinical observations on nausea, chills, back pain, headache, and other untoward reactions were made at frequent intervals. The magnitude of the febrile response was expressed quantitatively as a fever index calculated by the method of Beeson.[6] Using graph paper with 10 by 10 divisions to the half-inch, 20 divisions on the ordinate were taken to equal one hour and 10 divisions on the abscissa to equal 1 degree Fahrenheit. The control temperature was taken as the base line and the area above this line described by the temperature curve was measured with a compensating polar planimeter. This area expressed in square centimeters is the fever index. It represents an expression of both height and duration of fever.

Ten venous blood samples were drawn in the following time sequence: (1) control, (2) 20 minutes, (3) 40 minutes, (4) 60 minutes, (5) 120 minutes, (6) end of infusion, (7) 60 minutes after end of infusion, (8) 120 minutes after end of infusion, (9) 180 minutes after end of infusion, (10) 240 minutes after end of infusion. Blood for chemical determinations was immediately placed in an ice bath and kept refrigerated until analyzed.

Free fatty acid was determined by a method devised for this study and based in part on the method described by Davis.[7] Three milliliters of 95 per cent ethyl alcohol was added to 2 ml. of serum buffered by 1.0 ml. of 0.2 M phosphate buffer (pH 5.0). This mixture was extracted 3 times with 3 ml. portions of petroleum ether (B.P. 30° to 60° C.) by shaking for 1 minute, centrifuging, and removing the supernatant with a syringe and needle. The combined petroleum ether extracts were evaporated to dryness in a bath

gradually raised to 70° C. and the residue was taken up in 2 ml. of alcohol, heated for 1 minute in a water bath at 90° C., and titrated with 0.02 N aqueous sodium hydroxide using thymol blue as the indicator. Carbon dioxide-free air was bubbled through the alcohol solution during the titration. Blanks, in which water was used in place of serum, were run with each set of determinations. The results are expressed as milliequivalents of free fatty acid per liter.

Total fatty acid was determined by the method of Smith and Kik,[8] with slight modifications. Optical density was read against distilled water in microcuvettes in a Coleman Junior colorimeter at 700 mμ. Serum lysozyme determinations were done by a modification of the method described by Kerby.[5]

The same emulsions which were used for the study on human subjects were used in a companion study in rabbits. A six-hour pyrogen test was performed on 12 rabbits, using both emulsions. The dose levels used, expressed as grams of fat per kilogram body weight, were 1 Gm./kg., 1.7 Gm./kg., and 2.9 Gm./kg. Tests were performed every third day for six test days. Each rabbit received each emulsion at 3 dose levels according to a Latin square design. The febrile response was expressed as a fever index calculated by the method of Beeson.[6]

RESULTS

Fever (Fig. 1).—The mean maximum febrile response with the HSPH emulsion was 1.6° F. above the control value and occurred within one hour after the end of the infusion. The mean maximum febrile response with Lipomul was 2.3° F. above the control value and occurred in the third postinfusion hour. With neither emulsion was the mean rectal temperature back to the control level by the fifth postinfusion hour.

TABLE I. FEBRILE RESPONSE OF HUMAN SUBJECTS TO INTRAVENOUSLY INFUSED FAT EMULSIONS, RATE OF REMOVAL FROM THE BLOOD OF INTRAVENOUSLY INFUSED FAT EMULSIONS, AND RATE OF IN VITRO CLEARING OF FAT EMULSIONS BY POSTHEPARIN PLASMA*

SUBJECT	FEVER INDICES		REMOVAL INDEX (% FALL IN OPTICAL DENSITY/HOUR)		IN VITRO CLEARING INDEX (% FALL IN OPTICAL DENSITY/HOUR)	
	HSPH	LIPOMUL	HSPH	LIPOMUL	HSPH	LIPOMUL
E. M.	351	404	29	39	51	80
K. G.	189	357	19	25	21	58
A. S.	244	180	29	56	58	88
J. K.	104	299	9	18	70	97
A. T.	45	484	20	27	19	37
G. S.	125	77	14	29	17	43
J. M.	208	233	11	8	72	70
H. P.	533	29	18	36	2	4
Mean	225	258	18.6	29.8	38.8	59.6

*See text for explanation of indices.

In Table I are presented the fever indices for each infusion using the mean of the 20 and 40 minute temperatures as the base line and using all temperature records up to eight hours after the infusion. Analysis of vari-

ance reveals no significant difference in fever index between the two emulsions, or between the first infusions and second infusions. The correlation coefficient between the fever indices for the two emulsions in the individual subjects was $r = -0.49$, which is not statistically significant ($P > 0.05$).

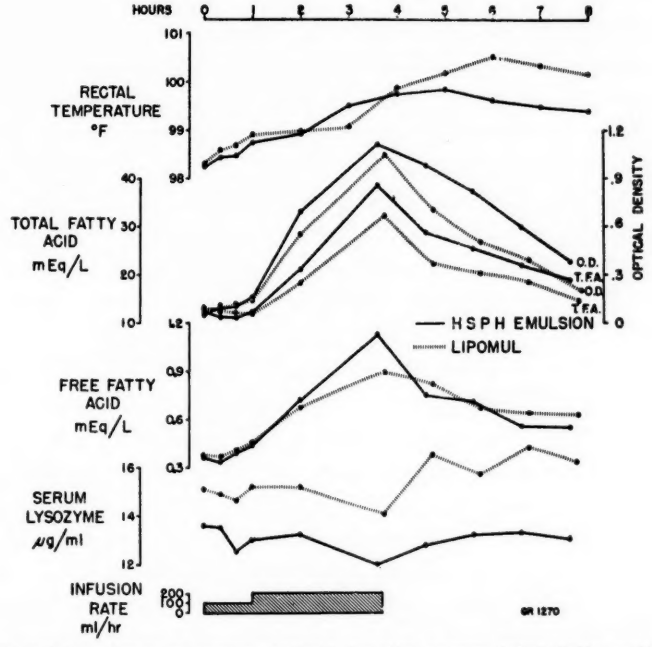

Fig. 1.—Rectal temperature, serum optical density, serum total fatty acid concentration, serum free fatty acid concentration, and serum lysozyme concentration of human subjects receiving intravenous infusions of fat emulsions.

Optical Density (Fig. 1).—The optical density of the serum showed only a slight rise during the first hour of the infusions with both emulsions at which time the infusion rate was 30 drops per minute or about 90 ml. per hour. During the remainder of the infusion when the drop rate was 60 per minute (approximately 180 ml. per hour) the optical density rose rapidly, reaching its peak at the end of the infusion. The optical density at the end of the infusion was significantly higher ($P < 0.05$) with the HSPH emulsion than with the Lipomul emulsion. Furthermore, the rate of return toward the control value was more rapid with the Lipomul than with the HSPH emulsion, although in neither case had the mean value returned to the control level by four hours after the end of the infusion.

The rate of decline in the optical density following the cessation of infusion was calculated as a Removal Index in which the change in optical density was expressed as per cent fall per hour according to the formula:

$$\frac{(\text{optical density at end of infusion—optical density at X hours})\ (100)}{(\text{optical density at end of infusion})\ (\text{number of hours})}$$

Only values of optical density above 0.200 were used in calculating this index and as many hours after the infusion, up to 4, were used as had values above

this. Table I presents the removal indices for the individual infusions. The mean difference between the removal indices for the two emulsions is statistically highly significant ($P < 0.01$) and the correlation between the removal indices for the two emulsions for individual subjects is also highly significant ($r = 0.85$, $P < 0.01$).

In Vitro Clearing of Emulsions by Postheparin Plasma.—The rates at which the emulsions were cleared in vitro by postheparin plasma, obtained from the subjects 15 minutes after intravenous injection of 0.5 mg. per kilogram of heparin, are presented in Table I. As with the in vivo removal indices (Table I), there was a highly significant difference between the two emulsions, the Lipomul emulsion being cleared in vitro and removed from the blood in vivo at the more rapid rate. The correlation coefficient between the rates of clearing of the two emulsions by the individual subjects was highly significant ($r = 0.89$, $P < 0.01$). However, the correlations between rates of in vitro clearing and in vivo removal were not significant. When the difference in clearing rates in vitro and in removal rates in vivo were expressed as a ratio, these ratios were highly correlated.

Total Fatty Acid (Fig. 1).—In general the total fatty acid levels followed the optical density values quite closely. However, during the slow rate of infusion in the first hour, the total fatty acid levels fell slightly while the optical density values were rising slightly. The peak total fatty acid level occurred at the end of the infusion, and then gradually fell. With neither emulsion did the mean value return to the control level by the fourth hour after the end of the infusion. The decline was more rapid with the Lipomul emulsion.

Free Fatty Acid (Fig. 1).—Free fatty acid levels paralleled the total fatty acid levels during the infusion, showing a distinct peak at the end of the infusion. After the end of the infusion the fall in free fatty acid concentration was gradual and in almost all instances was still elevated above the control level four hours after the end of the infusion. This persistent elevation was present even in most instances in which the optical density and total fatty acid level had returned to control levels. The serum from the end-of-infusion samples from 9 infusions were centrifuged at 14,000 r.p.m. for 30 minutes at 5° C., and the free fatty acid level was determined on the clear infranatant. In every instance the values were within a few hundredths of a milliequivalent of those for the corresponding whole lipemic serum.

Lysozyme (Fig. 1).—The serum lysozyme concentration showed no significant variation during the infusions and a small decrease at the end of the infusion. This end-of-infusion drop is attributable to inhibition of the lysozyme reaction by lipemia because when determinations were made on the clear infranatant of the serum obtained by centrifugation at 14,000 r.p.m. for 30 minutes at 5° C. the values were higher than those of the corresponding whole lipemic sera. In the postinfusion period there was a small elevation of lysozyme concentration with the Lipomul, but not with the HSPH infusions.

Respiratory Rate, Pulse, and Blood Pressure (Fig. 2).—Respiratory rate rose slightly and progressively during the course of and following the infusions. There was no significant difference between the emulsions in this respect. Pulse rate essentially followed the febrile curves, the elevation with the Lipomul emulsion being greater and more prolonged than with the HSPH emulsion. During the course of the infusion a slight but significant fall in pulse rate occurred with the Lipomul but not with the HSPH emulsion. Systolic blood pressure did not change significantly with the HSPH emulsion and with the Lipomul emulsion showed a small but significant drop during the infusion returning promptly to the control level when the infusion ended. Diastolic blood pressure showed a small rise following the infusion with both emulsions.

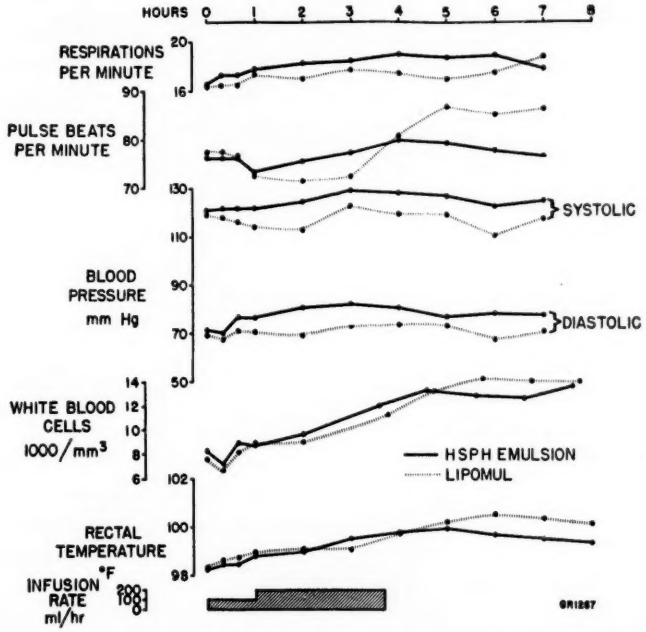

Fig. 2.—Respiratory rate, pulse rate, blood pressure, white blood cell counts, and rectal temperatures of human subjects receiving intravenous infusions of fat emulsions.

White Blood Cell Count (Fig. 2).—A well-marked leukocytosis occurred with each infusion of both emulsions. A slight and transient decrease in leukocyte count during the first hour of the infusion occurred in most instances. The peak leukocytosis occurred shortly after the end of the infusion and continued at this level for the duration of blood sampling, i.e., four hours after the end of the infusion. The pattern and magnitude of the leukocytic response was similar with the two emulsions.

Side Reactions Other Than Fever.—Shaking chills occurred during the onset of the fever in 3 instances with Lipomul infusions and one with HSPH emulsion. In all instances the chills subsided within one-half hour.

Nausea occurred in association with 4 of the HSPH emulsion infusions and 6 of the Lipomul infusions. In 2 instances with the Lipomul infusions it was accompanied by vomiting. There was no regularity in the time of occurrence of the nausea although in most instances it was most severe near the end of the infusion and for several hours thereafter.

Headache was noted twice with the HSPH infusions, once with the Lipomul infusions.

Flushing, mainly in the blush areas, occurred 4 times with the HSPH emulsion infusions and 2 times with the Lipomul infusions. In all instances the flushing began within a few minutes after starting the infusion and subsided within one hour.

Pain in the lumbar region of the back occurred 3 times, all with Lipomul infusions. In 2 instances in which the pain was mild it commenced between one and two hours after starting the infusion. In the one instance in which the pain was severe it began within a few minutes after starting the infusion. In all cases the pain subsided promptly. In two subjects the infusion was stopped for 10 and 20 minutes, respectively, during which time the pain subsided and the infusion was recommenced without recurrence of the pain.

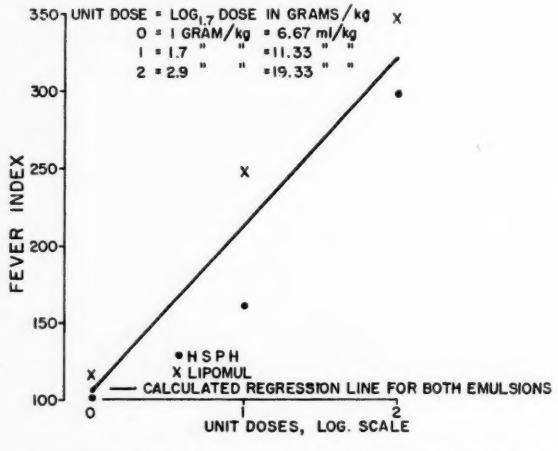

Fig. 3.—Febrile response of rabbits to intravenous injection of various doses of two different fat emulsions.

In one subject the infusion of Lipomul emulsion was stopped after 16 minutes because of a severe allergic-type reaction. One week later this same subject received an infusion of HSPH emulsion with no untoward reactions of any kind. The chemical and hematologic data for both infusions on this subject have been excluded from the presentation because one infusion was incomplete.

Febrile Response in Rabbits.—Although the mean fever index with the Lipomul emulsion was higher than with the HSPH emulsion, the difference was not statistically significant. This result is similar to that obtained in the studies on human subjects. There was a progressive rise in the mean fever index on

successive days of testing from the first to the fourth day with leveling off at this time. This effect was statistically highly significant. The difference between rabbits was statistically significant, in contrast with the result in the studies on human subjects where the differences between subjects were not significant. However, this is probably due to the much larger number of tests done on the rabbits. The validity of the testing procedure in rabbits is attested by the factorial analysis[9] which showed that the regression of fever index on logarithm of dose is very highly significant and does not depart significantly from linearity; and that the separate dose-response curves do not depart significantly from parallelism (Fig. 3).

The reason for separating the febrile response tests in rabbits by 3 days was that some rabbits were found to have elevated rectal temperatures as long as 48 hours after a preceding test with the highest dose of either emulsion.

DISCUSSION

None of the biochemical or hematological changes studied correlated well with the incidence or severity of fever. The one biochemical measurement which showed some tendency to be correlated with fever was the rate of removal of the infused fat from the blood as measured by optical density and total fatty acid concentration. However, even in this instance the correlation was not significant when measured as a correlation coefficient between removal rate and fever index for individual subjects. On the other hand, the rate of removal was different for the two emulsions, and in the individual subjects there was good correlation between the rates of removal of the two emulsions. In other words, there were differences between the two emulsions in the rates at which they were removed from the blood and there were differences between individuals in the rates at which they removed emulsions, and these differences occurred consistently. In the in vitro studies with post-heparin plasma the deturbidification or "clearing" of added fat emulsion was more rapid with the Lipomul emulsion than with the HSPH emulsion, thus corresponding to the relative rate of removal of the two emulsions in vivo. This finding suggests that a mechanism comparable to the heparin-clearing phenomenon participates in vivo in the process of removal of infused fat emulsion from the blood. However, the absolute rates of clearing in vitro were not correlated with the in vivo removal rates in the individual subjects. The reason for the difference between the two emulsions in in vivo removal and in in vitro clearing is not apparent. The emulsifying agents were the same but differed in amounts used. The most important difference is in the oils.

The tendency for a correlation to exist between clearing rate and febrile response suggests that the febrile response is not due to the presence of the unaltered fat particles in the blood but is in some way related to a change in their physicochemical state and/or their removal from the blood. This view is supported by the studies in rabbits made by Lambert et al.[10] in which it was found that emulsions prepared with an emulsifying agent which markedly retarded the removal of the fat from the blood produced no febrile response.

Optical density of the sera during the lipemic phase showed good correlation with total fatty acid content. Considering only values of optical density above 0.200, the correlation coefficient between optical density and total fatty acid content was +0.85 for the HSPH emulsion and +0.89 for the Lipomul. During the period of slow infusion, optical density rose slightly while total fatty acid fell slightly. This suggests that the amount of nonturbid lipid in the serum was falling and turbid lipid was rising during this period. On the other hand, neither optical density nor total fatty acid changed very much during the period of slow infusion, indicating that the fat emulsion was being removed from the blood almost as rapidly as it was being infused. During the period of rapid infusion both optical density and total fatty acid rose sharply, indicating that removal from the blood was slower than the rate of infusion. If these deductions are valid, they indicate that the maximal capacity for total removal of these fat emulsions from the blood lies between 100 and 200 ml. of emulsion per hour or between 15 and 30 Gm. of fat per hour.

Previous studies have demonstrated that small amounts of free fatty acid are present in fasting rat serum and that alimentary lipemia or heparin injection produces a marked rise in free fatty acid concentration.[2] It has also been shown that in vitro lipemia clearing by postheparin plasma is attended by marked increase in free fatty acid with a concomitant decrease in esterified fatty acid.[11] These findings suggest that one of the normal mechanisms of removal of turbid fat from the blood is lipolysis of neutral fat with release of free fatty acid which becomes bound to protein and is thus transported to the tissues. It is not yet possible to evaluate the extent to which such a mechanism participates in normal fat metabolism. In the present study the concentration of the free fatty acid paralleled that of the total fatty acid during the infusion, remaining at control levels during the slow rate of infusion, and reaching a peak of about three times the control level at the end of the infusion. Thus lipemia due to infusion of fat emulsions is associated with elevation of free fatty acid just as is alimentary lipemia. The elevation of serum levels of free fatty acid cannot be attributed to the free fatty acid content of the emulsions which were found to be 5.07 meq. per liter for the HSPH emulsion and 9.64 meq. per liter for the Lipomul emulsion. At the end of the infusions the elevation of total fatty acid concentration in serum was approximately 24 meq. per liter which represents about 0.05 volumes of emulsion in one volume of serum. This concentration of emulsion in serum would give about 0.5 meq. per liter of free fatty acid for the Lipomul emulsion and 0.25 meq. per liter for the HSPH emulsion on the basis of their contents of preformed free fatty acid. Since considerably higher concentrations of free fatty acid were found in the serum, it is reasonable to conclude that additional free fatty acid was released in vivo. Furthermore, essentially all of the free fatty acid was in the nonturbid portion of the serum. After the end of the infusion the free fatty acid did not return toward control levels as rapidly as did the total fatty acid, usually remaining elevated four hours after the end of the infusion even in

those instances in which the total fatty acid had returned to the control level. In the Lipomul infusions the free fatty acid levels remained elevated longer in the high febrile reactors than in the low febrile reactors, but this did not occur with the HSPH emulsion. Thus, there is no clear indication that free fatty acid levels were correlated with febrile response.

The studies on serum lysozyme were prompted by the observation of Kerby[5] that injured leukocytes release lysozyme, and the studies of Bennett and Beeson[3] demonstrating that a thermogenic substance is released by lysis of leukocytes. A standard curve was made each day using crystalline egg-white lysozyme, and the position of this curve was found to vary considerably. Therefore, the absolute values for serum lysozyme are probably not reliable, but the relative changes during the course of the infusion probably are. Although there was some tendency for the lysozyme to rise following the Lipomul infusions, the correlation with febrile response was poor. These results do not support the hypothesis that leukocyte injury may be involved in the thermogenic response to fat emulsions. However, since the reliability of serum lysozyme concentration as an index of leukocyte injury is not established, the hypothesis warrants further study.

SUMMARY

Intravenous infusions of emulsions of cottonseed oil and of olive oil were given to normal human subjects. The magnitude of the febrile response was not well correlated with changes in optical density, total fatty acid, free fatty acid, or lysozyme of the serum, or with changes in leukocytosis. Differences between the two emulsions and between individual subjects were demonstrated in regard to rate of removal of the emulsions from the blood and rate of clearing of the emulsion in vitro by postheparin plasma.

Free fatty acid concentration of serum rose during the lipemia induced by intravenous infusion of fat emulsion. The increased content of free fatty acid was shown to be in the nonturbid portion of the serum.

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HEMORRHAGIC LIPEMIA: A DERANGEMENT OF FAT METABOLISM

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REPEATED heavy bleedings cause visible lipemia in rabbits,[1] accompanied by a large increase in the plasma neutral fat and a smaller increase in lecithin and cholesterol. Little change in the lipid content of erythrocytes occurs.[2] During recovery, the lipemia disappears long before the number of erythrocytes returns to normal. Although rabbits do not normally develop alimentary lipemia, they do after moderate bleedings.[2, 3] Phenylhydrazine or distilled water injected intravenously also causes lipemia.[3, 4] Hemorrhagic lipemia can be produced in guinea pigs as well,[5] but not in dogs.[6] Feigl[5, 7] found that lipemia often developed in patients after heavy blood loss or with pernicious anemia. According to Froehlich,[8] most human anemias are associated with hypolipemia, although some patients with pernicious anemia have an elevated neutral fat level. In pernicious anemia, the duration of alimentary lipemia is prolonged.[9]

The genesis of hemorrhagic lipemia is unknown and has been frequently a subject of speculation. According to Starup,[4] the removal of cells, rather than of plasma, is responsible for its development. This differs from Johansen's[10] observation that lipemia develops despite the reinjection of removed cells; and from Ellerman and Meulengracht's[11] finding that the reinjection of the removed serum prevents the development of lipemia. Edelman[12] believes the disturbance is localized on the cellular level, since intestinal absorption is undisturbed. Milne[13] suggests that incomplete peripheral oxidation is responsible, and Sakai[3] and Edelman[12] indicate the possible involvement of serum lipase. Fishberg[14, 15] correlates lipemia with the decrease in plasma albumin during bleeding. The lipids are presumably mobilized from the yellow marrow.[6, 16] The participation of the central nervous system is indicated, since transection of the spinal cord at the level of the fourth thoracic prevents the development of hemorrhagic lipemia.[17]

The objectives of this study of hemorrhagic lipemia were to learn more about its etiology and to use an "endogenous derangement of fat metabolism" (it develops without introducing any foreign material into the animal) as a tool to elucidate more details about normal fat metabolism.

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MATERIALS AND METHODS

Hemorrhagic lipemia was produced mainly in male rabbits weighing about 2,000 to 3,000 grams. Twenty-five to thirty milliliters of blood were removed daily by cardiac puncture for 7 to 10 days. The sera were analyzed ultracentrifugally at a density of 1.21 according to the method of Lewis and associates[18] as modified by Boyle and associates.[19] The flotation rates were expressed in $-S_{1.21}$.[18] The corresponding Gofman flotation rates are[20]:

$-S_{1.21}$ 0-20 = α lipoproteins
$-S_{1.21}$ 20-52 = S_f 2-10
$-S_{1.21}$ 52-74 = S_f 10-20
$-S_{1.21}$ 74-300 = S_f 20-100

The term "total lipoproteins," as used in this paper, refers to the sum of the groups just given.

The activity of clearing factor in vivo was followed by measuring the turbidity of the serum in a Coleman Jr. spectrophotometer at wave length 700 mμ. In vitro clearing activity was tested by incubating the serum with a cottonseed oil emulsion* and following the turbidity changes in the spectrophotometer. Clearing factor was purified by the method of Nikkilä.[21]

The clearing-enhancing capacity of sera was determined by incubating them with a mixture of postheparin dog or rabbit serum and oil emulsion. Clearing of each test mixture was observed for several hours. The enhancing capacity was obtained by comparing the clearing in each mixture with that of the control containing postheparin serum, oil emulsion, and saline. Paper-strip electrophoresis was carried out in Veronal buffer of pH 8.6 and $\Gamma/2 = 0.1$.

RESULTS

Ultracentrifugal Studies.—The marked increase in blood lipids during repeated bleedings prompted a study of the lipoproteins of experimental animals developing hemorrhagic lipemia. Fig. 1 shows the average daily lipoprotein values of eight rabbits. A marked and consistent elevation of the $-S_{1.21}$ 74-300 lipoproteins is the most striking change. The other groups did not change consistently. Occasionally, they exhibited considerable fluctuation. The total lipoproteins rose almost parallel to the $-S_{1.21}$ 74-300 group. The elevated lipoproteins returned to a normal level a few days after bleedings were discontinued. The actual ultracentrifugal changes of the $-S_{1.21}$ 74-300 group in one of eight rabbits are presented in Fig. 2. Similar increases in twelve additional rabbits were found during occasional ultracentrifugal tests.

Seven daily cardiac punctures without drawing any blood did not produce similar changes in two rabbits. Regular daily bleedings of five rabbits were taken and the washed erythrocytes were reinjected into the animals for 7 to 10 days. Lipemia with the same ultracentrifugal changes in the blood developed in four rabbits, although somewhat more slowly. The injection of 30 ml. of physiologic saline after daily bleedings did not change the course or development of hemorrhagic lipemia in three rabbits. Daily bleedings and the reinjection of about 70 to 80 per cent of the citrated plasma failed to prevent the development of visible lipemia and ultracentrifugal changes in the blood of three animals.

*Generously donated by Dr. H. C. Meng, Vanderbilt University, Nashville, Tenn.

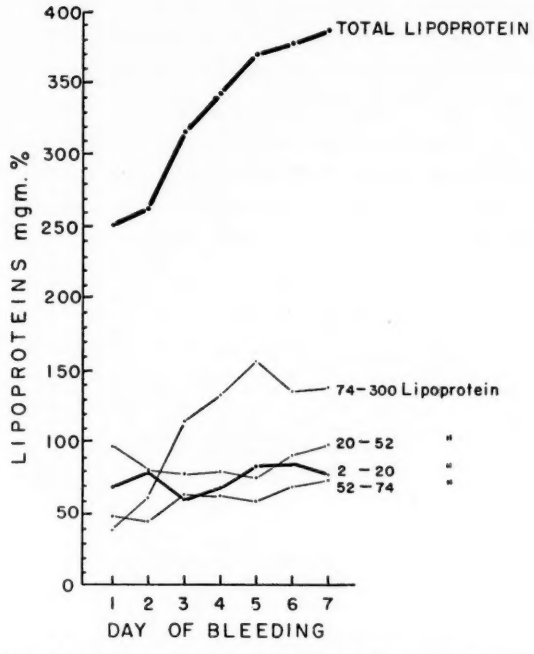

Fig. 1.—Concentration of different groups of lipoproteins during development of hemorrhagic lipemia.

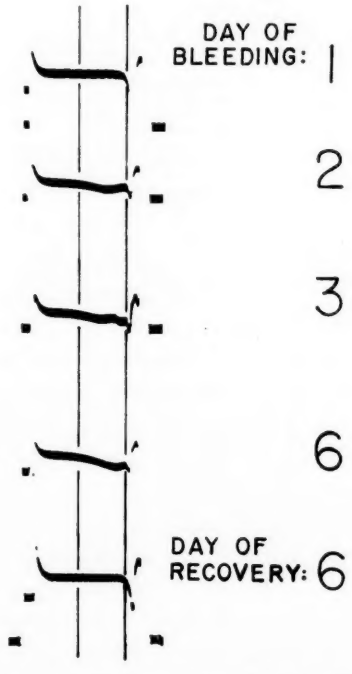

Fig. 2.—Rabbit No. A340. Changes in ultracentrifugal patterns of $-S_{1.21}$ 74–300 lipoproteins (between the two vertical lines) during bleeding and after recovery. Photographs taken upon reaching maximum speed.

The intravenous injection of 1 mg. (0.5 ml.) of heparin three times daily failed to alter hemorrhagic lipemia in three rabbits. The effect of heparin given at the height of hyperlipoproteinemia in nine rabbits was also investigated. It seemed to cause a slight and transient drop in most of the lipoproteins. However, evaluation is difficult since, in rabbits with such a severe anemia, the removal of the 10 ml. of blood needed for ultracentrifugal analysis initiates certain regulatory and counterregulatory mechanisms which alter the lipoprotein spectrum.

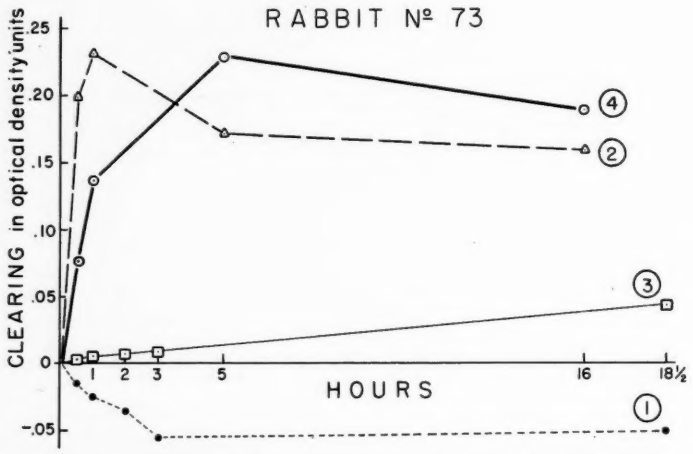

Fig. 3.—Clearing activities of post heparin (1 mg., I.V.) whole sera and purified clearing factors of a lipemic animal and of same animal after recovery. Curve *1*, 0.5 ml. postheparin lipemic serum and 1.0 ml. oil emulsion. Curve *2*, 0.5 ml. postheparin serum after recovery and 1.0 ml. oil emulsion. Curve *3*, 0.5 ml. purified (after removal of lipoproteins) clearing factor from lipemic animal, 1.0 ml. oil emulsion, and 0.5 ml. normal rabbit serum. Curve *4*, 0.5 ml. purified (after removal of lipoproteins) clearing factor after recovery, 1.0 ml. oil emulsion, and 0.5 ml. normal rabbit serum.

Clearing-Factor Studies.—Because of the intimate relationship between lipemia clearing factor and lipemias of different etiology, the production and activity of the clearing factor were investigated in rabbits with hemorrhagic lipemia. As reported in preliminary communications,[22, 23] hemorrhagic lipemia alters both the clearing mechanism and the ability of the animals to produce clearing factor. One milligram of heparin injected intravenously produces little or no clearing of lipemia. Upon incubating the postheparin sera with an oil emulsion, no clearing occurs, and the mixture gradually becomes turbid. Postheparin serum alone also becomes turbid on standing. (Clotting time increases in postheparin blood samples.) Clouding in the postheparin samples does not occur if the blood is oxalated or citrated, probably because of the prevention of insoluble calcium soap formation. If decalcified postheparin plasma of lipemic rabbits is incubated with an oil emulsion, a very slight clearing of the emulsion takes place. The same rabbits, however, show marked clearing-factor activity if the same amount of heparin is injected before the bleedings are started or after recovery from lipemia. Similar results were obtained when a purified clearing factor was prepared from the serum of animals with hemorrhagic

lipemia and from the same animals after recovery. Fig. 3 presents one of the five experiments comparing the activities of the two clearing factors. Experiments on nonlipemic rabbits served as controls.

Animals which were bled to cause anemia, but not extensively enough to develop lipemia, showed normal clearing-factor activity after the injection of heparin. The feeding of fat at this stage caused marked lipemia which cleared readily after the injection of heparin.

Clearing-Enhancing-Capacity Experiments.—If normal plasma or serum is incubated with a clearing-factor substrate mixture, clearing is enhanced. This may be due to at least three different mechanisms; the fatty-acid-acceptor capacities of serum albumin and of lipoproteins, and the presence of cofactor. The enhancing capacity reflects mainly the fatty-acid-accepting capacity of the serum as indicated by the following observation: The enhancing capacity of dog serum is greater than that of rabbit serum which, in turn, is greater than that of rat serum. On the other hand, in in vitro clearing tests with rat postheparin serum, the test mixture starts to cloud after moderate initial clearing. Using rabbit postheparin serum, the same clouding phenomenon occurs

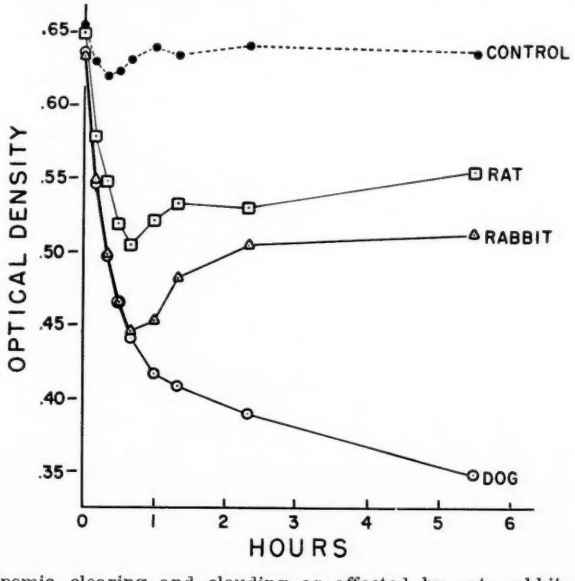

Fig. 4.—Lipemia clearing and clouding as affected by rat, rabbit, and dog sera. Test mixtures contain 0.5 ml. rat postheparin serum, 1.0 ml. oil emulsion, and 0.5 ml. of normal rat, rabbit, or dog serum. Saline used in control.

after greater initial clearing. It practically never takes place with canine postheparin serum. Since clouding is usually due to calcium soap formation, which occurs after the serum albumin is saturated with fatty acids, any change in its occurrence would indicate an altered fatty-acid-accepting capacity. A comparison of clearing and clouding, as affected by normal dog, rabbit, and rat sera, is shown in Fig. 4. It is apparent that dog serum is more efficient in accepting fatty acids than rabbit serum which, in turn, is more efficient than

rat serum. This seems to be due to a qualitative rather than quantitative difference in the serum albumin of the different species, since dogs have a lower concentration of serum albumin than either rabbits or rats.[24]

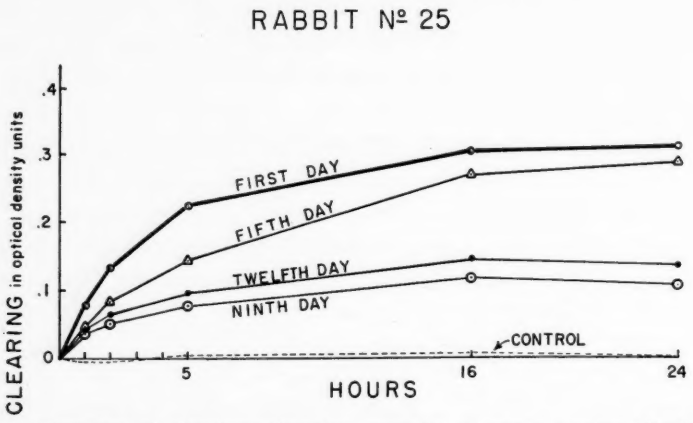

Fig. 5.—Decrease of clearing-enhancing capacity of serum during bleedings. Each test mixture contains 0.5 ml. rabbit serum (after removal of lipoproteins) obtained on indicated day of bleeding, 1.0 ml. oil emulsion, and 0.5 ml. normal purified rabbit clearing factor. Control mixture is same as first day's mixture excluding normal rabbit serum.

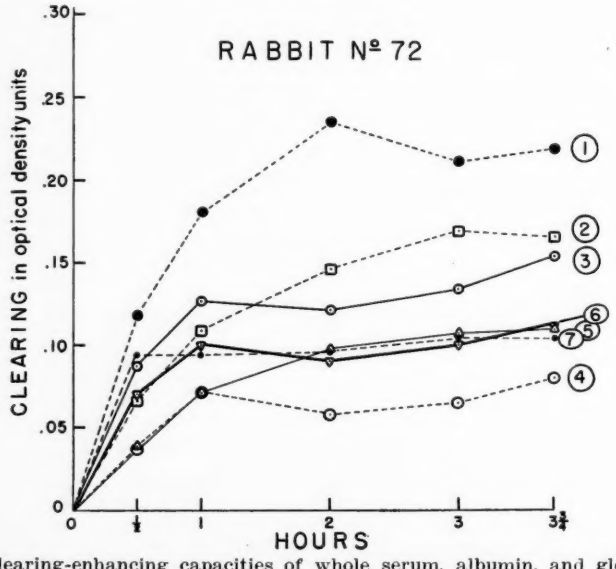

Fig. 6.—Clearing-enhancing capacities of whole serum, albumin, and globulins in normal rabbit and in same animal after development of hemorrhagic lipemia. Curve *1*, 0.5 ml. dialyzed rabbit whole serum, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor. Curve *2*, serum albumin corresponding to 0.5 ml. of above serum, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor. Curve *3*, serum globulins corresponding to 0.5 ml. of the above serum, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor. Curve *4*, 0.5 ml. whole serum from same rabbit after development of lipemia, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor. Curve *5*, serum albumin corresponding to 0.5 ml. of above lipemic serum, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor. Curve *6*, serum globulin mixture corresponding to 0.5 ml. of above lipemic serum, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor. Curve *7*, control: 0.5 ml. saline, 1.0 ml. oil emulsion, and 0.5 ml. rabbit clearing factor.

Since clearing-factor production is altered in hemorrhagic lipemia, the clearing-enhancing capacity of the hemorrhagic lipemic sera was investigated. In all fifteen animals studied, there was a consistent decrease in the enhancing

capacity of the serum during bleeding. The decrease varied from animal to animal due to individual variation and to the method's lack of quantitative accuracy. However, the findings were qualitatively well reproducible. The maximal decrease generally corresponded to the appearance of visible lipemia, although strongly decreased enhancing capacity, simultaneous with clear serum, occurred occasionally. Fig. 5 shows the decrease of the enhancing capacity of one animal during bleedings.

To learn more about the decrease in the enhancing capacity of the serum, albumin and globulins were separated by 50 per cent saturation with $(NH_4)_2SO_4$ and the enhancing capacity of the fractions compared after dialysis. In five out of six experiments, the enhancing capacity of the albumin was decreased during bleedings (three of these experiments were done in duplicate). There was a smaller and less consistent decrease in the enhancing capacity of globulins in three of six animals during bleeding (Fig. 6).

Electrophoretic Studies.—No appreciable change in albumin concentration, as determined by paper-strip electrophoresis, occurred during bleeding. Some of the globulins, however, especially α_2 and β, were increased (Table I).

TABLE I. PAPER ELECTROPHORETIC CHANGES IN RABBIT SERA DURING DEVELOPMENT OF HEMORRHAGIC LIPEMIA

DISTANCE FROM STARTING POINT IN CM.	DAY OF BLEEDING 1ST	5TH	9TH	12TH
	OPTICAL DENSITY UNITS AT 575 Mμ*			
-2†	.057	.077	.053	.049
-1†	.121	.140	.122	.122
0	.165	.192	.155	.150
1	.162	.183	.138	.136
2	.137	.192	.133	.121
3	.134	.181	.157	.114
4	.130	.185	.155	.133
5	.132	.205	.155	.132
6	.162	.252	.194	.197
7	.153	.198	.172	.153
8	.163	.201	.167	.151
9	.182	.269	.243	.179
10	.194	.262	.224	.237
11	.168	.198	.187	.168
12	.209	.297	.303	.228
13	.450	.520	.610	.472
14	.09	.131	.108	.08
15	.097	.112	.064	.077

*Bromphenol blue was eluted from paper strips with 2 ml. of 0.1 per cent Na_2CO_3.
†Toward cathode.

DISCUSSION

Hemorrhagic lipemia is accompanied by a marked increase of the total and of the $-S_{1.21}$ 74-300 lipoproteins. Other lipoproteins are not characteristically altered although, occasionally, quite large day-to-day changes are observed. This is not surprising in animals in which the dynamic equilibria of protein and fat metabolism are so greatly disturbed, since the lipoprotein level and constitution of normal individuals also vary considerably.[25, 26] The simultaneous changes in the $-S_{1.21}$ 74-300 group and in the total lipoproteins

indicate an absolute and basic derangement of the lipoprotein metabolism rather than a rearrangement within the different groups, although a rearrangement might also take place simultaneously.

The accumulation of lipoproteins in the course of their metabolism may reflect increased mobilization or synthesis, decreased utilization, an intermediary block, or a combination of these factors. Increased mobilization is certainly a factor, since fat depots are almost devoid of fat in hemorrhagic lipemia. This increased mobilization is mediated through the central nervous system, either directly or indirectly (stress?), as indicated by Mill[17] who showed that hemorrhagic lipemia does not develop after the transection of the spinal cord at the level of the fourth thoracic. Unfortunately, extremely little is known about the utilization of lipids in hemorrhagic lipemia, although this should be a profitable field for investigation.

According to the recent concept of lipoprotein metabolism,[27] lipoproteins undergo a continuous transformation from large, neutral, fat-rich, low-density molecules to smaller, cholesterol-rich, and high-density ones. In the course of this transformation, clearing factor acts as a physiologic agent. If the cause of hemorrhagic lipemia were a simple increase in the mobilization of fats or a decrease in their utilization, an over-all, and more or less uniform increase in all of the different groups of lipoproteins would be expected. In hemorrhagic lipemia, a "bottleneck" is encountered in the stepped-up conversion of lipoproteins between the $-S_{1.21}$ 74-300 and the $-S_{1.21}$ 52-74 groups. Since this particular point in the conversion of lipoproteins corresponds to the site of attack for the clearing factor,[28] the production and activity of clearing factor was studied in these animals. The production of clearing factor was found to be impaired after the injection of heparin. Clearing-factor production is dependent on heparin, a precursor protein, and a tissue factor.[29, 30] Its action also requires the presence of a cofactor[29] and of albumin.[31, 32] Lack of heparin is probably not responsible for the disturbance, since its administration during bleedings fails to prevent the appearance of hemorrhagic lipemia. Heparin is not very effective either, when injected into an animal with hemorrhagic lipemia. It is not known whether there are any changes in the precursor protein level of the blood in hemorrhagic lipemia, but the fact that precursor protein is an α lipoprotein[29, 30] and that, in general, the α lipoprotein level of the blood does not change consistently, would decrease the likelihood of the precursor protein as the cause of the changes. What changes, if any, take place with regard to the tissue factor is not known. The exact role and importance of the cofactor are undecided; therefore, its possible participation in the etiology of the condition is difficult to evaluate.

Albumin is required in the process of clearing and it acts as fatty acid acceptor, accepting and transporting the liberated fatty acid from the low-density lipoproteins. In hemorrhagic lipemia, the plasma albumin may be loaded with fatty acid; therefore, it may accept only a limited amount during clearing. The possibility may exist that the amount of albumin decreases

during bleeding, and that this is responsible for the decreased capacity to accept fatty acids. However, paper-strip electrophoresis revealed no appreciable decrease in the albumin fraction during bleeding. Sometimes an increase was noticed, due possibly to an increase in the α_1 globulins which are often indistinguishable from the albumin peak. In the course of bleeding, an increase in the α_2 and β globulins was often noted, a finding which supports the data of Werner.[33] The impaired transport of fatty acids probably represents one of the important changes in hemorrhagic lipemia. A similar situation exists in lipoid nephrosis.[34] There are some experimental indications that the globulins do not enhance clearing as efficiently in rabbits with hemorrhagic lipemia as they do in normal animals. The cause of this change is not clear. Undoubtedly, the presence of a large concentration of $-S_{1.21}$ 74-300 lipoproteins may decrease clearing,[30] but the same change also occurs after removing the large low-density lipoproteins by floating them in the ultracentrifuge. The possibility of a circulating anticlearing factor cannot be ruled out.

Hemorrhagic lipemia is probably accompanied by profound changes in the hormonal balance of the organism, but few concrete facts are known about this. Perhaps the most interesting is the observation that hemorrhagic lipemia is accompanied by hyperglycemia.[12] Some of the hormonal changes are being studied in this Laboratory.

SUMMARY

Hemorrhagic lipemia was produced in rabbits, and the resulting deranged fat metabolism was characterized by ultracentrifugal lipoprotein determinations, clearing-factor studies, clearing-enhancing-capacity experiments, and electrophoretic analyses. The total lipoprotein level as well as that of the $-S_{1.21}$ 74-300—Gofman's S_f 20-100—showed regularly a marked increase; the other groups of lipoproteins changed only irregularly and occasionally. Clearing-factor production is impaired in hemorrhagic lipemia. After the injection of heparin, sera from lipemic animals have less clearing activity than before lipemia or after recovery. The clearing-enhancing capacity of the plasma or serum of the bled animals is decreased. It is concluded that in hemorrhagic lipemia there is an increased mobilization of lipids, a block in the metabolism of lipoproteins, due possibly to altered clearing-factor production, and a decreased capacity of albumin to accept and transport fatty acids.

It is a pleasure to acknowledge the valuable technical assistance rendered by Mrs. V. M. Grant and Mr. E. F. Duchna.

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A METHOD FOR DETERMINING AERO-ALLERGEN CONCENTRATIONS WITH THE MOLECULAR FILTER MEMBRANE

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THE need for a simple, rapid, accurate method of determining the concentration of aero-allergens has long been recognized. As part of a broader interest in the effects of weather on disease, we planned to investigate the meteorological influence on the symptoms of patients with ragweed sensitivity. From the onset it seemed imperative that diurnal fluctuation of ragweed concentration should be studied and correlated, if possible, with the meteorological data. The available techniques for determining ragweed pollen variation such as the widely used gravity sampler of O. C. Durham[1] did not offer the precision and quantitative results we required.

It has been suggested that the ideal method of measuring allergenic air contamination should employ a volumetric sampling unit.[1] Available techniques of pollen sampling have been ingenious compromises with this ideal and the reality that no such practical apparatus existed.[1-3] Recently, however, a device known as the molecular filter membrane became available. This filter, paper thin, and containing 5 times 10^7 pores per square centimeter actually removes all particles greater than 0.2 μ from a liquid or gas passing through it.[4] It has been used previously in studying water pollution[5] and contamination of air with pathogenic organisms,[6] but never previously, to our knowledge, has it been applied to the problem of aero-allergens.

In this paper we wish to outline the technique we adapted for obtaining accurate estimates of the diurnal variation of aeroallergens, and to suggest a simple technique for routine daily pollen determinations by a modification of the more complex apparatus.

MATERIALS AND METHODS

In essence the equipment consists of a battery of molecular filter membranes, connected to an air pump, and controlled by electrically operated valves. In order to obtain samples automatically throughout each day a timing device and switch were added.

Figs. 1 and 2 are photographs of the apparatus used. Fig. 3 is a schematic diagram of the equipment. There are six heads (*a*) attached by threaded metal tubing to electrical valves (*b*). In each of the heads is a molecular filter membrane. The valves are connected by pressure tubing and a manifold to an electrically operated vacuum pump (*e*). Inserted in the pressure manifold between pump and valves is a limiting flow orifice (*f*) for control of the rate of flow of air through the filters. The valves (*b*) are connected by an electrical conduit (*g*) to a rotary switch (*h*) which is activated by current from the timer (*i*) flowing into the solenoid (*j*). The timing device is also connected to the vacuum pump (*e*). The

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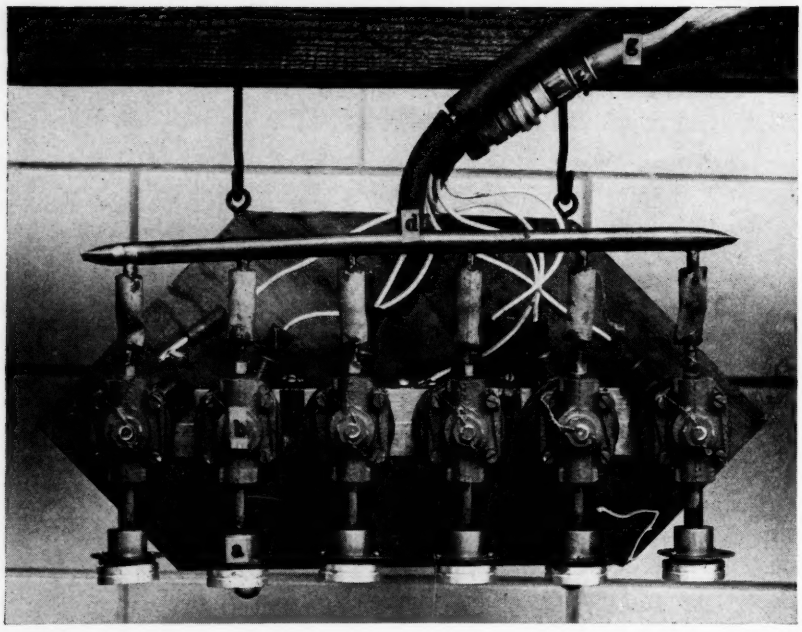

Fig. 1.—Six heads (*a*) each containing a molecular filter membrane, and electrically operated valves (*b*). See text.

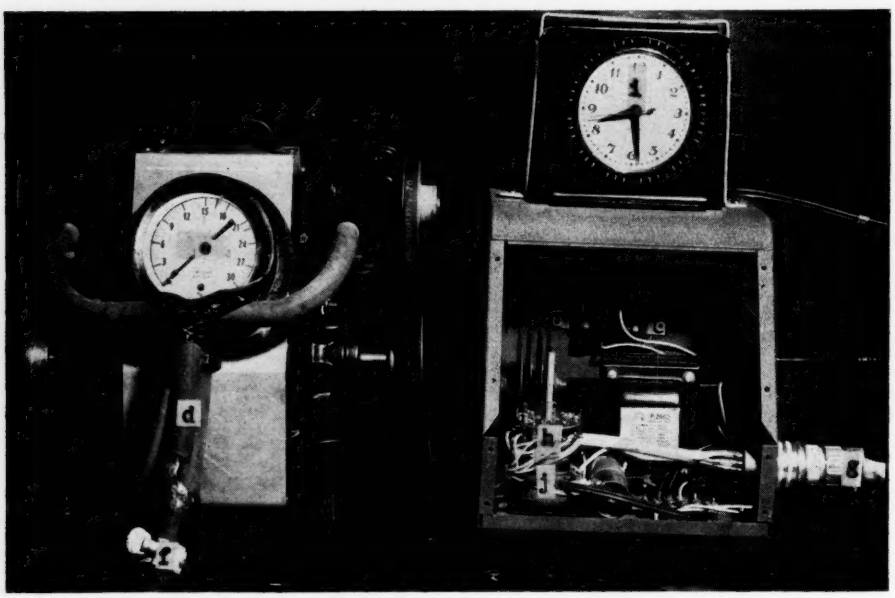

Fig. 2.—Vacuum pump timing device, and electric switch. See text.

timer was so adjusted that it permitted flow of current during the first thirty minutes of every four hours. When the current flowed, the switch moved to a new position, the corresponding electrical valve was opened, and the vacuum pump was turned on. When the current stopped, at the end of thirty minutes, the vaccum pump was turned off and the valve was closed. Four hours later this cycle was repeated with the next valve being opened. At the end of each 24-hour period there were six filters, each of which contained all of the aero-allergens of particle size greater than 0.2 μ in the volume of air which had passed through them. By repeatedly calibrating our apparatus with a Sargent wet test meter it was ascertained that the pump, with interposed limiting flow orifice, worked at the rate of 9 L. per minute or 270 L. per 30 minutes (approximately 10 feet^3 per 30 minutes).

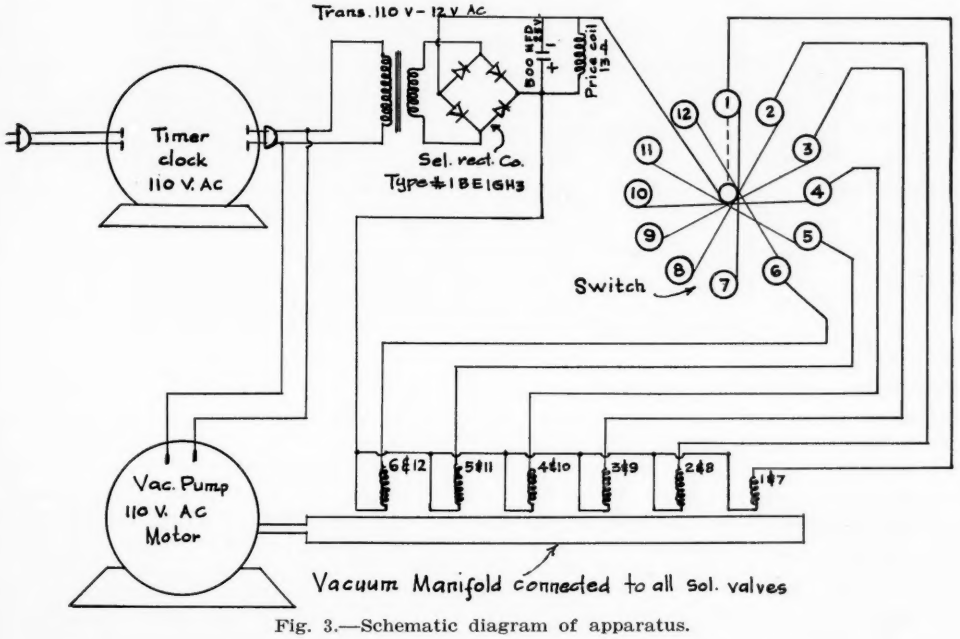
Trans. 110 V - 12 V AC
Timer clock 110 V. AC
Sel. rect. Co. Type # 1BE1GH3
Switch
Vac. Pump 110 V. AC Motor
Vacuum Manifold connected to all Sol. valves

Fig. 3.—Schematic diagram of apparatus.

It was necessary to demonstrate that pollen collected on the molecular filter membrane while the pump was in operation did not fall off in the interval between the time a given sample was obtained and the time the molecular filter membrane was prepared for counting. That there was not appreciable loss was demonstrated by simultaneous determinations using two sets of apparatus placed side by side. The pollen on one membrane was counted immediately; the other membrane was left on its apparatus for 24 hours and then counted. A series of such determinations showed less than 7 per cent variation between the corresponding counts, the determination after 24 hours of weathering sometimes being slightly greater than the count obtained by preparing the mount immediately after sampling. Some of the differences may be due to slight variation of pollen concentrations at two points close together in space, and future investigations are planned to assess the uniformity of concentration of pollen even at great distances from the source of production.

At the end of each 24-hour period the heads (*a*) were unscrewed from the valves and the filters were removed and prepared for counting. Fig. 4 shows the equipment necessary for mounting the filters. The head, with the contained molecular filter membrane is shown at *1*. The membrane itself *2* is placed on a black background for contrast. Other equipment includes a 10 c.c. syringe *3* with No. 20 needle cut to ½ inch length and containing yellow petrolatum; a tuberculin syringe *4* with No. 25 needle bent to 90 degrees and filled with Calberla's fluid; an aluminum template *5* with a one-inch hole; ordinary glass microscope

slide and one inch square cover slip. The template was used to make a ring of petrolatum one inch in diameter on the glass slide, using the syringe and needle much as a fountain pen. Seven-hundredths of a milliliter of Calberla's solution was then measured into this ring with the aid of the tuberculin syringe, and evenly distributed by tilting the slide. The filter was then removed from the head, placed face up in the center of the petrolatum ring, and a cover slip placed on top. This formed a semipermanent mount. The Calberla's solution

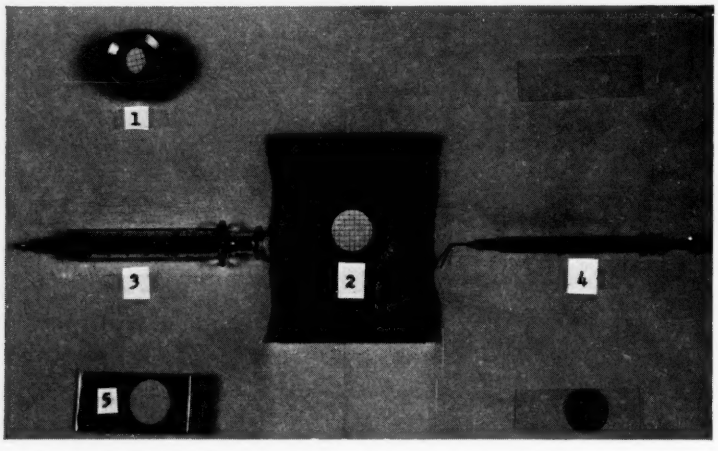

Fig. 4.—Equipment necessary for mounting the filters. See text for details.

was absorbed by the membrane from its undersurface, making it translucent and staining the pollen; the pollen was as easily counted by a microscope using transmitted light as on the ordinary gravity slide. Very little focusing was necessary because most of the particulate matter was contained in a single plane. It would have been possible to use reflected light as well, but such mounts lack permanence, and smaller particles such as Alternaria and smut are not as readily identifiable.

RESULTS

The apparatus as described enabled us to obtain a daily profile of the pollen concentration throughout most of the ragweed season in Ann Arbor, 1954. It is apparent that by simple modification of this basic apparatus more points on the daily profile might have been obtained, perhaps with increasing accuracy and flexibility. The details of our results are reported elsewhere[7]; characteristic figures are given in the following example which illustrates the advantages of this method of assay over the conventional gravity-sampling technique, at least

TABLE I

	GRAVITY	8:00 A.M.	12:00 A.M.	4:00 P.M.	8:00 P.M.	12:00 P.M.	4:00 A.M.
September 3	87	64	188	169	107	30	15
September 4	91	19	126	175	98	248	235

for our purposes. Table I shows results obtained on two consecutive days during which the gravity pollen determinations were similar. The profiles obtained with the molecular filter membrane, however, were quite different. On September 3, the count was low in the morning, rising during the midday and afternoon, only to fall very low at night and in the early morning. On September 4,

this same pattern was apparent for the day with somewhat different numerical values. Instead of falling at night, however, there was a sharp increase to very high levels which remained until the next morning. These fluctuations of concentration escape detection when 24-hour counts are obtained by the gravity sampler. It remains for future studies to determine how valuable these daily profiles of pollen fluctuation will be to the clinical allergist. Profiles of fluctuations of aero-allergen concentration obtained in this manner may aid in explaining allergic symptoms occasionally experienced by patients at night and in the early morning.

A simple modification of this basic idea is possible for routine pollen surveys. A small, relatively inexpensive air pump is available to which one of the previously described molecular filter membranes can be attached by a suitable head. This air pump can be allowed to run continuously for 12 or 24 hours, and the filter prepared and mounted much as described previously. The practicing allergist will then have a filter containing all of the pollen removed from a known volume of air, in a given time and place. He need not resort to hypothetical conversion factors[8] which have proved to be notoriously inaccurate,[9] or have nebulous results influenced greatly by the vagaries of wind and rain.[10]

SUMMARY

An apparatus is described for obtaining accurately the diurnal variation of airborne pollens. A simple modification of the complex equipment is suggested for routine pollen surveys.

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LABORATORY METHODS

A SIMPLE, RAPID PROCEDURE FOR THE ESTIMATION OF ALBUMIN AND ALPHA, BETA, AND GAMMA GLOBULIN IN SERUM

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CHARLESTON, S. C.

CHEMICAL methods which have been proposed in recent years for the determination of serum proteins and which have been designed to give results approximating those obtained by electrophoresis are based mainly on modifications and combinations of the following procedures: (1) estimation of the protein (albumin) remaining in solution after precipitation of globulins by methanol,[1] by 26 per cent sodium sulfate,[2-4] or by 28 per cent sodium sulfite,[5] (2) estimation of albumin plus alpha globulin as the protein remaining in solution after precipitation by the Howe technique[6] or modification thereof,[3-5] (3) estimation of gamma globulin as the protein precipitated under specified conditions by zinc sulfate,[7] sodium sulfate,[4] ammonium sulfate,[8] or saline ammonium sulfate.[5, 9]

In order to determine albumin and alpha, beta, and gamma globulin in serum by any combination of these methods, the total protein must also be estimated. Such combinations as have been proposed by Kibrick and Blonstein[4] and by Wolfson and associates[5] require four separate measurements of the serum sample, several troublesome filtrations or centrifugations, and four protein determinations—all of which detract from the usefulness of these methods in the routine clinical laboratory. The method to be described here is based upon observations by Butler and co-workers[10, 11] and also by Rapoport and colleagues[12] in regard to precipitation of serum proteins by phosphate solutions. The proposed procedure requires only one measurement of the serum sample, no filtrations or centrifugations, and protein concentrations are determined by simple turbidimetric measurements.

METHOD

Reagents.—

Stock phosphate solution: 3.33 M, pH 6.5. Add 2,268 grams of potassium phosphate, monobasic ("Sorensen's Potassium Phosphate"-KH_2PO_4), to about 4,000 ml. of solution containing 335 grams of sodium hydroxide. Shake or stir until completely dissolved. Cool to room temperature, then dilute to 5,000 ml. or add water until the final weight of the solution is 6,675 grams.

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Dilute phosphate solutions: No. 1. Weigh 1,235 grams of stock phosphate solution into a 1,000 ml. volumetric flask. Dilute to the mark with distilled water.

No. 2. Weigh 1,000 grams of stock phosphate solution into a 1,000 ml. volumetric flask. Dilute to the mark with distilled water.

No. 3. Weigh 785 grams of stock phosphate solution into a 1,000 ml. volumetric flask. Dilute to the mark with distilled water.

No. 4. Weigh 625 grams of stock phosphate solution into a 1,000 ml. volumetric flask. Dilute to the mark with distilled water.

Procedure.—Set up five colorimeter tubes marked B (blank), 1, 2, 3, and 4. Into Tube B measure 10 ml. of distilled water and into the other tubes measure 10 ml. of the correspondingly numbered dilute phosphate solutions. Now measure 1.0 ml. of serum and 1.5 ml. of water into a small test tube. Add 7.5 ml. of stock phosphate solution, allowing it to flow directly into the diluted serum. Invert the tube 5 or 6 times to mix, then transfer 1 ml. of the mixture to each of the previously prepared colorimeter tubes, again allowing the serum-phosphate mixture to flow directly into the solutions in the colorimeter tubes. Mix the contents of each tube thoroughly by rotating or inverting the tube, but avoid vigorous shaking. Allow to stand for about fifteen minutes, then measure the optical density of Tubes 1, 2, 3, and 4 at 650 mμ, using Tube B to set the spectrophotometer at zero optical density (100 per cent T).

Calculations.—

O. D. Tube No. 1	× F = Total protein
O. D. Tube No. 1—O. D. Tube No. 2	× F = Albumin
O. D. Tube No. 2—O. D. Tube No. 3	× F = Alpha globulin
O. D. Tube No. 3—O. D. Tube No. 4	× F = Beta globulin
O. D. Tube No. 4	× F = Gamma globulin

Calibration.—The factor F used in the above calculations must be determined for the particular type of spectrophotometer used. Obtain a sample of clear, normal human serum. Determine the total protein content of this serum by any accurate, convenient method. Carry the serum through the previously given procedure, using the blank and Tube No. 1 only (in triplicate). Divide the total protein of the serum by the average O. D. of Tube No. 1 to give factor F. For convenience, a calibration card showing per cent protein corresponding to various colorimeter readings may be prepared.

All turbidimetric measurements reported in this paper were made with a Coleman Model 11 Spectrophotometer fitted with a special adapter to take 22 mm. test tube cuvettes. Recent experiments with a Bausch & Lomb Spectronic 20 colorimeter and one-half inch test tube cuvettes indicate that one-half quantities of serum and reagents may be used throughout this procedure with equally satisfactory results. With some instruments it may be necessary to obtain protein values from a calibration curve rather than by use of a factor.

EXPERIMENTAL

The turbidity of serum-phosphate mixtures depends not only upon the amount of protein present and the concentration of phosphate, but also upon the way in which the mixtures are prepared. In our early experiments we added varying amounts of water and of "stock" phosphate solution to the serum samples. Turbidity measurements obtained in this way were found to be affected by the rate of addition of phosphate solution and by shaking the solutions while the phosphate was being added. Even when these conditions were carefully standardized so that reproducible results could be obtained in most cases, an occasional serum sample was found to flocculate so rapidly that the turbidity measurements were unreliable. Attempts to improve results by changes in the order of addition of serum, water, and phosphate, or by use of "protective colloids" were unsuccessful.

In all of these early experiments, the final dilution of serum was 1:100 (1:10 with 0.9 per cent sodium chloride, then 1:10 with water and phosphate). Later, we added water and then phosphate solution to the serum sample so as to produce a final phosphate concentration of 2.5 molar and a serum dilution of 1:10. One milliliter aliquots of the resulting suspension were then transferred to 10 ml. portions of phosphate solutions of various concentrations (made by dilution of stock phosphate with water). Turbidity measurements made on these mixtures were found to be reproducible and sufficiently stable for reliable results to be obtained in all cases. This procedure for preparation of the serum phosphate mixtures is therefore recommended in this method and has been used in all experiments reported in this paper.

Butler and associates[10, 11] studied the solubility of plasma proteins in phosphate solutions and observed breaks in the solubility curve indicative of successive precipitation of plasma protein fractions. The phosphate concentration range for precipitation of various fractions of horse plasma was given as follows: fibrinogen, from 0.7 to 1.1 molar; euglobulin, from 1.2 to 1.6 molar; pseudoglobulin, from 1.5 to 2.5 molar; albumin, from 2.4 to 3.0 molar. With human plasmas, the break between euglobulin and pseudoglobulin was not observed. However, Rapoport and co-workers[12] used phosphate solutions for the fractionation of serum proteins of children and concluded that the fraction precipitating below 1.6 molar phosphate was chiefly gamma globulin. Other fractions were not characterized and no electrophoretic comparisons were made.

In view of the results obtained by the above investigators, we have used the turbidity of serum phosphate mixtures as a measure of protein precipitation and have compared the turbidity, at or near the suggested phosphate concentrations, with the concentration of serum protein fractions as determined by the chemical method of Wolfson and associates.[15] The phosphate concentrations selected and the serum protein fractions precipitated are as follows: 3.0 molar—total protein; 2.5 molar—total globulins; 2.0 molar—beta and gamma globulins; and 1.65 molar—gamma globulin. These are the final phosphate concentrations in Tubes Nos. 1, 2, 3, and 4, respectively, as prepared in the proposed method.

Forty samples of serum submitted to this laboratory for routine sodium and potassium determinations, without regard to diagnosis or clinical condition, and twenty normal samples collected from healthy medical students have been analyzed for total protein and for alpha, beta, and gamma globulins by the proposed turbidity method and also by the chemical method of Wolfson and associates.[5] The results have been plotted and tested by Pearson's product-moment correlation coefficient.

$$r_{xy} = \frac{\Sigma\ (xy)}{N\sigma_x\sigma_y}$$

Where x and y are the deviations of the observed values from their respective means, N is the total number of cases, and σ_x and σ_y are the standard deviations of the respective means. Perfect correlation is indicated by an r of 1.0 whereas

an r of 0 indicates no correlation. For each determination the standard error of estimate of the proposed phosphate turbidity method (the method of Wolfson and co-workers[5] being considered as reference) has been tested as follows:

$$S_y = \sigma_y \sqrt{1 - r^2}$$

where σ_y is the standard deviation as before and r is the correlation coefficient.

RESULTS

1. *Total Protein* (Fig. 1).—Results obtained by the proposed phosphate turbidity method and by the chemical method of Wolfson and colleagues[5] show good agreement. With 60 serum samples, a mean value of 7.1 grams per 100 ml. was obtained by each method. Good correlation is shown by an r of 0.87 and a standard error of estimate of 5.5 per cent at the mean.

2. *Albumin* (Fig. 2).—The correlation is good, r being 0.97 and the standard error of estimate 4.7 per cent at the mean.

3. *Total Globulin* (Fig. 3).—Good correlation is shown by an r of 0.93 and a standard error of 8.4 per cent at the mean. In general the turbidity values were slightly higher than the chemical ones.

4. *Alpha Globulin* (Fig. 4).—The correlation is poor, r being 0.53 and the standard error of estimate 17.0 per cent. The values obtained by the turbidity method were almost uniformly higher than those by the chemical method.

5. *Beta Globulin* (Fig. 5).—The correlation in general was poor, r being 0.71 and the standard error of estimate 11.9 per cent at the mean.

6. *Gamma Globulin* (Fig. 6).—The correlation between the two methods was good as shown by an r of 0.97 and a standard error of 11.0 per cent at the mean.

DISCUSSION

A statistical comparison of results obtained in the determination of serum proteins by the proposed phosphate turbidity method and by the chemical method of Wolfson and associates[5] indicates that the two methods may be expected to give similar results for total protein, albumin, total globulins, and gamma globulin. However, considerable discrepancy between the two methods may be expected in the case of alpha and beta globulins. It is interesting to observe that Popper and co-workers[13] have reported poor correlation between these same two protein fractions as determined by the chemical method in comparison with electrophoretic results. They suggest that a part of the discrepancy may be due to the fact that lipid and carbohydrate moieties which migrate with the alpha and beta globulins in electrophoresis are not recorded in the chemical method. Preliminary experiments on ether-extracted sera indicate that these factors are recorded in the phosphate turbidity method and may be partly responsible for the discrepancies which we have found in the determination of alpha and beta globulins. It seems reasonable to suppose, therefore, that the results obtained in the determination of alpha and beta globulins by the phosphate turbidity method may be found to correlate more closely with electrophoresis than with the chemical method.

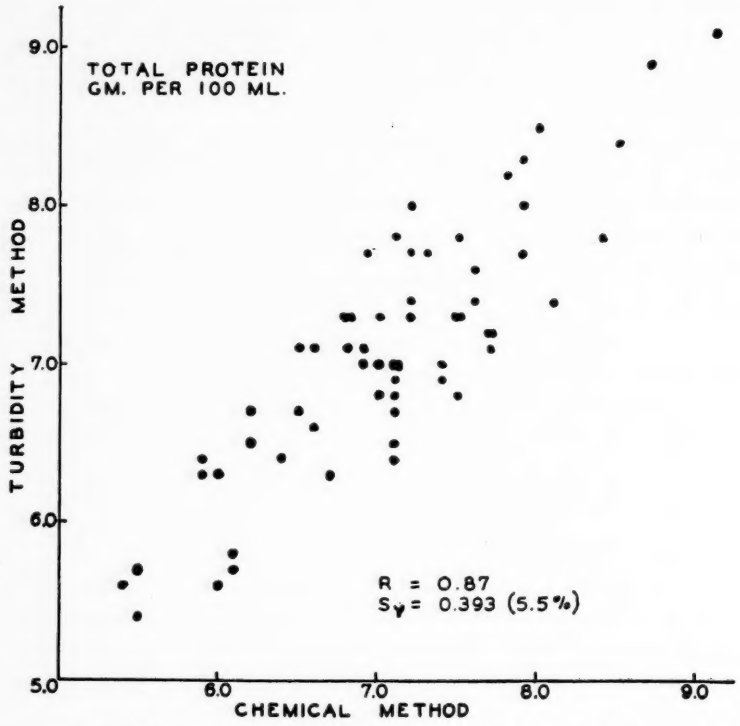

Fig. 1.—Comparison of total protein concentration as determined by chemical and turbidity methods.

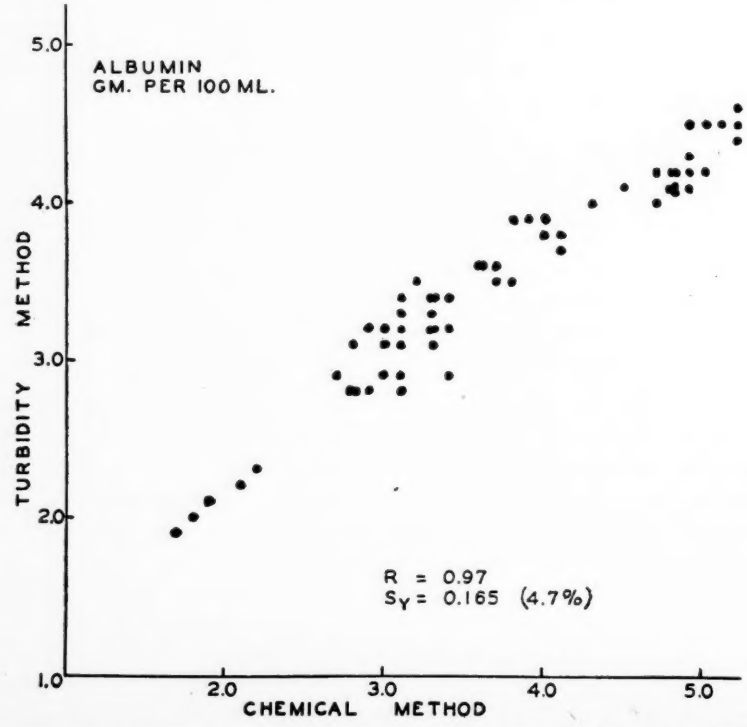

Fig. 2.—Comparison of albumin concentration as determined by chemical and turbidity methods.

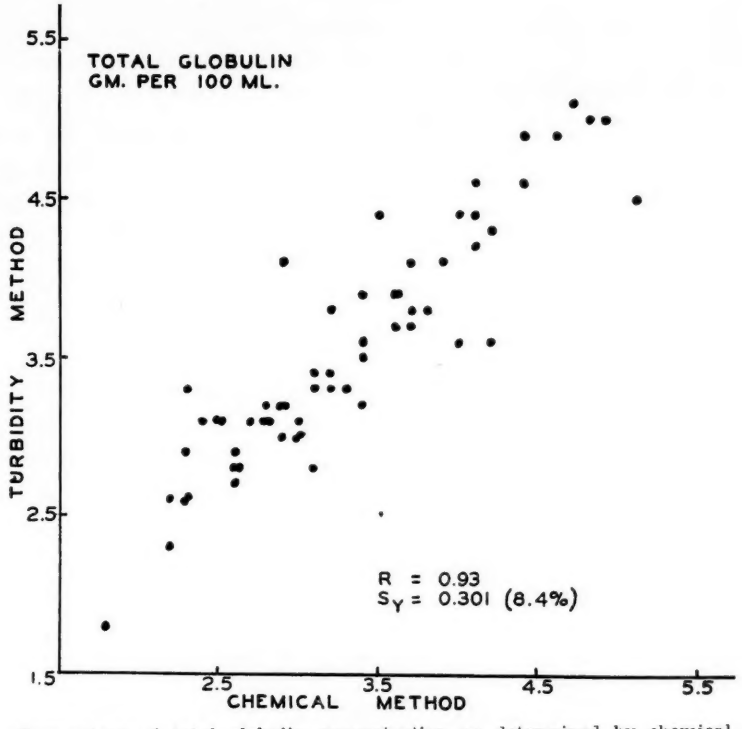

Fig. 3.—Comparison of total globulin concentration as determined by chemical and turbidity methods.

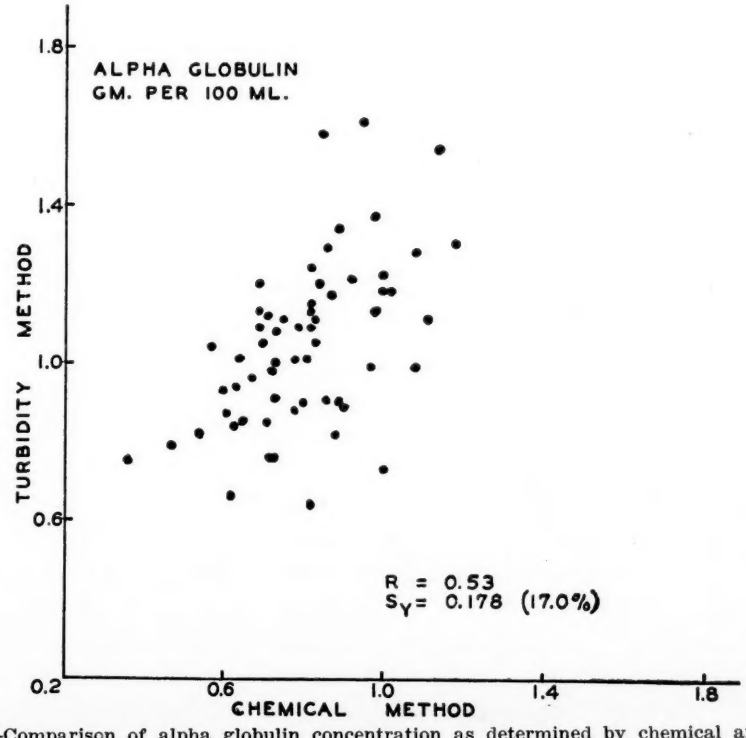

Fig. 4.—Comparison of alpha globulin concentration as determined by chemical and turbidity methods.

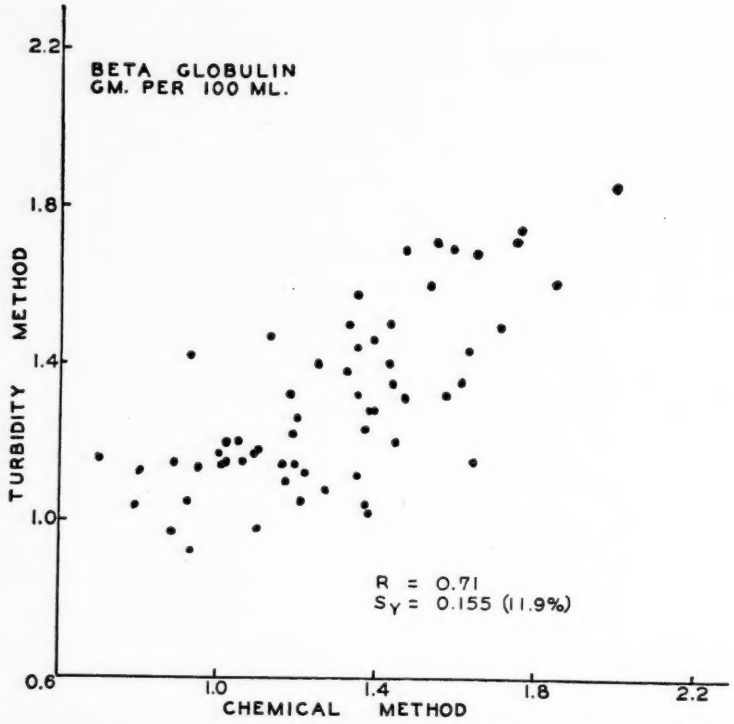

Fig. 5.—Comparison of beta globulin concentration as determined by chemical and turbidity methods.

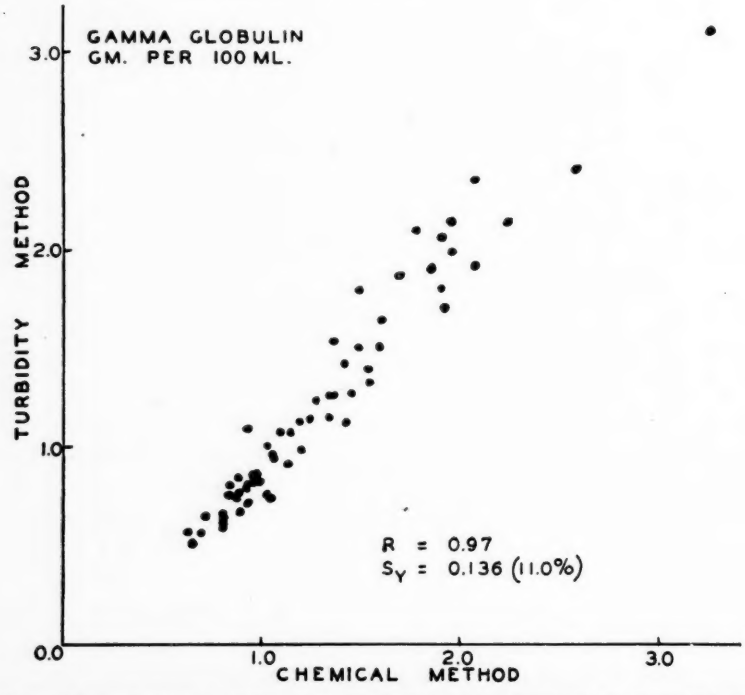

Fig. 6.—Comparison of gamma globulin concentration as determined by chemical and turbidity methods.

It is not suggested here that the change in turbidity of serum phosphate mixtures that occurs with any given increase in phosphate concentration is the result of precipitation of any single protein fraction as defined by electrophoresis. In common with other chemical precipitation methods there is probably an appreciable overlap in so far as precipitation of the various protein fractions is concerned. However, errors resulting from incomplete precipitation of one fraction apparently are nullified by partial precipitation of the next fraction. Furthermore, we believe that such errors are minimized in the proposed method because of the high dilution of serum used.

SUMMARY

A simple method for the estimation of serum albumin and alpha, beta, and gamma globulin has been described. The method is based upon the measurement of turbidity produced when serum is mixed as directed with phosphate solutions of specified concentrations. Sixty serum samples have been analyzed by the proposed method and by the more elaborate chemical method of Wolfson and associates.[5] A statistical comparison of the results is presented and discussed.

We wish to thank Miss Anne McCraw for assistance in making the chemical determinations and Mrs. Mary E. Ivester for preparation of the charts and typing the manuscript.

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ON THE PREPARATION OF SODIUM CHROMATE–Cr^{51} FOR RED CELL VOLUME DETERMINATIONS

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THE application of chromium[51] to the determination of red cell and plasma volumes has been described in a series of excellent papers by Gray, Sterling, and their associates.[1-5] These workers have shown that chromate–Cr^{51} is eminently suitable for determining erythrocyte volumes. Its radiation characteristics are amenable to scintillation counting; in addition, the binding of chromate to erythrocytes is essentially irreversible, obviating the difficulties normally encountered in erythrocyte volume determinations using radioactive phosphorus. For plasma volumes, chromic chloride–Cr^{51} provides the considerable advantage of eliminating the use of a foreign protein as in the I^{131} labeled albumins.

The preparation of chromate–Cr^{51} from chromic oxide, and the subsequent reduction of chromate to chromic ion, has already been described.[1] Since chromic chloride of high specific activity has recently been made available,[6] it was considered desirable to find a simple rapid method for the conversion of chromic chloride to sodium chromate. Such a method would provide a means whereby chromium could be readily available in both chromic and chromate forms for either plasma or erythrocyte volume determinations. Of the methods available for the chromic-chromate conversion, the most promising one appeared to be the chromic-chromite-chromate sequence. This paper describes the conditions for the facile conversion of chromic chloride to sodium chromate using aqueous alkaline hydrogen peroxide as the oxidant.

EXPERIMENTAL

The following procedure is applicable to chromic chloride as received from the Oak Ridge National Laboratory or to chromic chloride prepared from chromium metal by dissolution in hydrochloric acid.

One millicurie of chromic chloride-Cr^{51}, received from the Oak Ridge National Laboratory, in 0.4 ml. of 2.82 N hydrochloric acid, was diluted with water to 10 ml.; 5 ml. of this solution was taken for conversion to sodium chromate. The sample of chromic chloride-Cr^{51} was put into a 50 ml. beaker; the beaker was then placed in another vessel so that ice could be packed around the 50 ml. beaker. After cooling the solution below 10° C., 1 N sodium hydroxide (1.35 ml. total) was added dropwise, with stirring. A light blue precipitate appeared, which dissolved on addition of more sodium hydroxide. Only the minimum amount of sodium hydroxide necessary to effect conversion of chromic chloride to sodium chromite was used. It is imperative that the solution be kept cold during the addition of sodium hydroxide; at elevated temperatures, products are formed

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which do not dissolve on the further addition of sodium hydroxide. After the addition of sodium hydroxide had been completed, 30 per cent hydrogen peroxide was added dropwise until the blue color changed to a yellow color. Excess hydrogen peroxide was then removed by boiling. The solution was adjusted to pH 7.4 with 0.1 N hydrochloric acid and filtered to remove traces of insoluble matter; it was then ready for use in labeling erythrocytes.

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SPECTROPHOTOMETRIC ANALYSIS OF UNCONTAMINATED BLOOD FOR OXYHEMOGLOBIN

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A NUMBER of methods of evaluating the oxygen content of blood from its color changes have been used in the past.[1-9] Among the more recent of these are the adaptations to the commercial Beckman spectrophotometer[10, 11] and the various oximeters.[12, 13] Such methods are more rapid than the manometric technique and their simplicity permits continuous or very frequent samplings; moreover, the lack of interference from inhalation anesthetics gives such methods a unique advantage under special circumstances. At times, however, accuracy and reliability have been diminished to attain such advantages.

One of the questions arising from these various studies is whether it is possible to obtain satisfactory results by using the optical properties of blood, or whether variations are inherent in the light-absorbing properties of different hemoglobins and oxyhemoglobins. After analysis of some of the sources of error in spectrophotometric techniques of various kinds, we obtained data bearing on this point which may be useful with respect to any photometric procedure and evolved a rapid simple method of increased accuracy.

METHOD

Blood was drawn in syringes lubricated with petrolatum jelly, the dead space of which was filled with heparin-fluoride solution. The blood was frozen in a bath of dry ice and acetone, thawed in a room-temperature water bath and placed in spectrophotometer cuvettes anaerobically. A Beckman Quartz Spectrophotometer, model DU, was used with standard 1 cm. Corex cuvettes and commercially available spacer inserts to alter the light path length.* For most physiologic purposes arteriovenous [$(A\text{-}V)_{O_2}$] or other oxygen difference is desired, and this value is given by a single reading of venous blood optical density against a blank of simultaneously collected arterial blood; such samples may be expected to differ only in gas content except under unusual circumstances. Equally simple is the procedure of reading a test sample against a portion of the same blood which has been saturated with oxygen by rotating for several minutes in an oxygen-filled syringe; however, two readings with their attendant errors are necessary to calculate $(A\text{-}V)_{O_2}$ in this way, and it is employed only to measure absolute oxygen content.

Freezing was carried out in polyvinyl plastic tubing of 3.5 mm. bore which is readily available as disposable blood transfusion tubing.† It fits syringe tips and needle adapters tightly but easily. About 1.5 ml. of blood is needed to fill the cuvette and this volume is contained in a 35 cm. length of tubing. A sufficient quantity of blood for duplicate analysis was placed in each length of tubing, about 70 cm., and the two were treated simultaneously.

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In vessels of these dimensions, about 30 seconds are necessary for freezing and an equal time for thawing. In practice, the process was a continuous one for preparing two blood samples and required no timing.

The tubing was filled with mercury to prevent contact of the blood sample with air; preliminary washing with CO_2 removed O_2 absorbed in the interior wall of the tubing. Fig. 1 illustrates the mercury reservoir and tubing during use. About 25 to 50 feet of tubing were connected to each of the two outlets of the reservoir so that two blood samples could be handled together. The sample syringe was agitated and its tip flushed out, after which it was attached directly to the plastic tubing filled with mercury, care being taken to prevent contact with air. Blood was forced into the tubing under slight positive pressure from the reservoir and the tubing clamped below the blood level. Another clamp was placed on the tubing at the syringe tip which was then detached; the blood-filled segment was wound about the clamps and submerged in the CO_2-acetone bath and, subsequently, in the water bath.

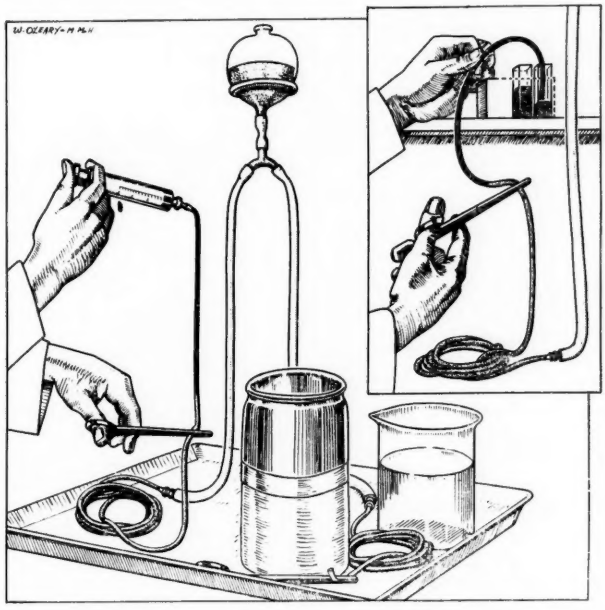

Fig. 1.—Apparatus for anaerobic hemolysis by freezing and thawing, showing method of filling and emptying the plastic tubing.

The free end of the tubing was cut off flush with the clamp by means of a scalpel to provide a clean aperture; this end was dried, unclamped, and inserted to the bottom of the spectrophotometer cuvette illustrated at an intermediate height between reservoir and table top. As the lower clamp was opened, mercury from the reservoir displaced hemolyzed blood into the cuvette.

Duplicate 1 cm. cuvettes were half-filled, *from the bottom up,* for each blood sample. A segment of blood 3 to 5 cm. in length was discarded. Prisms were then inserted into each cuvette to provide a light path length of 3 mm. (the most useful depth for arterial and mixed venous blood differences) or whatever depth was desired. Optical density readings between 0.100 and 0.400 were used. Five-tenths centimeter cuvettes were employed for comparison of normal arterial blood with a saturated sample. The cuvettes thus closed and having a protective layer of hemoglobin solution above the light path gave constant readings for at least an hour. Using the arterial or saturated blood samples as blank, the optical density was determined at a wave length of 660 mμ and a slit width of 0.05 mm.

The soiled tubing was cut off after clamping, the mercury deposited for cleaning and re-use, and the tubing discarded. The remainder of the mercury-filled tubing was then ready for the next sample.

RESULTS

1. *Completeness of Hemolysis.*—That complete hemolysis was produced by the method described has been confirmed on a number of bloods, including all those discussed hereafter. Hemolyzed samples were centrifuged for 30 minutes at 2,500 r.p.m., total pigment concentration (as cyanmethemoglobin) being determined (see following) before and after centrifugation. The values invariably differed by only a few thousandths of an optical density unit and are not given in detail; examples are shown in Table I, *B*. Likewise, the small amount of white precipitate obtained from 15 to 20 ml. of hemolyzed blood, consisting of red cell stroma, was resuspended in 2 to 2.5 ml. of the supernatant, and total pigment determinations were repeated. Somewhat lower values were always obtained, indicating that no hemoglobin-containing cells or fragments were present (Table I, *C*). The hemolyzed samples also yielded the same total pigment values as the fresh, untreated blood (Table I, *A*). Microscopic examination of 22 precipitates revealed no intact cells or pigmented fragments.

2. *Measurement of O_2 Contents.*—

TABLE I. EFFECT OF FREEZING ON CELL CONTENT OF BLOOD AND OF CELL FRAGMENTS ON APPARENT OXYHEMOGLOBIN CONTENT

SPECIMEN	OPTICAL DENSITY	
	BEFORE CENTRIFUGING	AFTER CENTRIFUGING
A. Fresh blood diluted 1:250, total pigment as cyanmethemoglobin		
1	.366	0
2	.204	0
3	.257	0
B. After freeze-thaw hemolysis		
1	.366	.367
2	.205	.204
3	.258	.258
C. B with cell fragments from 20 ml. resuspended in 2 ml.		
1	.360	.367
2	.204	.206
3	.255	.259
D. Freeze-thaw hemolyzed anaerobically, $(A\text{-}V)_{O_2}$ readings		
4	.106-.105	.106-.106
5	.194-.192	.193-.194
6	.220-.223	.221-.222
7	.287-.287	.286-.285
8	.343-.345	.344-.344
9	.392-.390	.390-.390

a. *Effect of cell fragments:* The solutions in the cuvettes were only slightly opalescent and showed no patterning of the suspended red cell stroma fragments. Under these circumstances the effect of opalescence was negligible, apparently identical in the test and blank samples. Table I, *D* shows the effect of centrifuging filled 5 mm. cuvettes, protected with an oil layer, immediately after taking the first readings. No significant change in optical density occurred although both test and blank (O_2-saturated) solutions were rendered perfectly

transparent. These results also confirm more directly the absence of pigment-containing fragments as indicated by the cyanmethemoglobin determinations after centrifugation.

b. *Correlation of optical and gasometric methods:* Analysis of a pair of blood samples for A-V oxygen difference in duplicate required about 5 minutes by the method described. The results of 34 such analyses are shown in Fig. 2 as a graph of the regression of optical densities (for 1 cm. light path length) on manometric A-V oxygen difference. Most of the blood samples were drawn during experimental and diagnostic cardiac catheterizations, mixed venous blood from the right side of the heart, arterial from brachial or femoral arteries. Some of the samples were peripheral venous blood partially or completely oxygenated by aeration. The manometric results were corrected for dissolved oxygen by estimating oxygen tension from a normal oxygen dissociated curve and multiply-

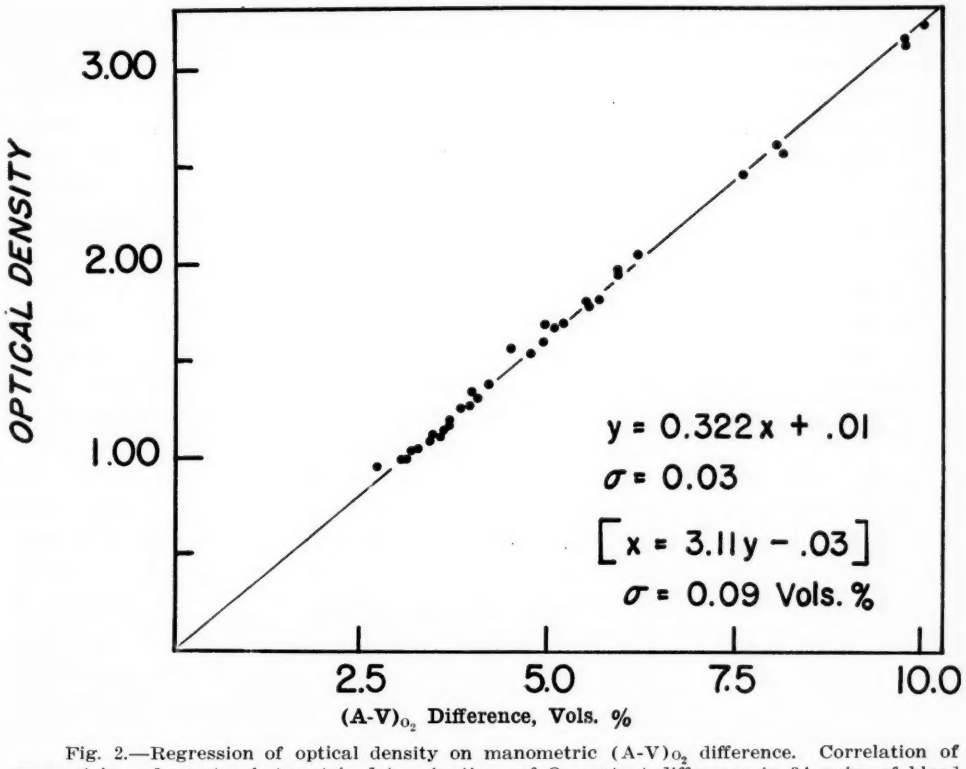

Fig. 2.—Regression of optical density on manometric $(A\text{-}V)_{O_2}$ difference. Correlation of gasometric and spectrophotometric determinations of O_2 content difference in 34 pairs of blood samples.

ing by the solubility factor of Sendroy and associates.[14] The regression coefficient was 0.322 optical density unit per volume per cent $(A\text{-}V)_{O_2}$ (millimolar extinction coefficient difference 0.722). The mean difference between the values obtained by the two methods was 0.01 vol. per cent ($\sigma = 0.09$ vol. per cent) when the observed regression coefficient was applied to the optical densities.

The data cover a range of differences from 2.71 to 13.28 vol. per cent (the latter not shown in the graph, but entered into the calculations) and wide

variations in pH, as in the aerated and peripheral venous bloods. Oxygen capacities varied from 10.64 to 30.25 vol. per cent. Both fasting and postprandial states are represented and show no difference. Many of the larger A-V differences were obtained during exercise of the subjects; no difference in the variability of these results was noted.

3. *Measurement of Percentage O_2 Saturation of Arterial Blood.*—Measurement of oxygen capacity of blood for calculation of per cent saturation was carried out on the hemoglobin solutions used for O_2 determination. The solutions were transferred from the cuvettes to tubes, and at some later time 2 ml. aliquots of the solutions were diluted to 500 ml. in triplicate and total pigment determinations carried out by the cyanmethemoglobin method[16] at a wave length of 540 mμ. The molar extinction coefficient derived from the comparison with oxygen capacity determinations by a modified[21] Van Slyke and Neill[18] method, corrected for dissolved oxygen with the factors of Sendroy,[14] was found to be 11.60, $\sigma = 0.09$, in 25 satisfactory analyses of whole blood and of saline suspensions of washed red cells in which no methemoglobin was found.

Methemoglobin present in the blood samples was not measured as reduced hemoglobin in the O_2 determination because this derivative was present in the blank in essentially the same concentration as the unknown sample. The mean value for resting arterial O_2 saturation in a group of normal subjects, obtained by combining the methods for O_2, total hemoglobin, and methemoglobin (see below) was 97.4 per cent, $\sigma = 0.9$, $n = 12$.

4. *Estimation of Other Pigments.*—This method of analysis of undiluted, uncontaminated blood may be adapted to methemoglobin determination using a wave length of 635 mμ and completely or largely oxygenated blood in a depth of 1 cm. The blood may be frozen in stoppered test tubes and centrifuged. The blank consists of a portion of the same hemoglobin solution obtained after freezing and thawing but placed in a cuvette containing a small crystal of potassium cyanide. Concentrations which can be determined accurately are from 0.05 to 0.7 vol. per cent capacity (0.023 to 0.3 mM per liter). In a group of 20 analyses of 10 bloods converted completely to methemoglobin by the potassium ferricyanide method of Austin and Drabkin,[20] diluted with water to a variety of concentrations and read as described, the regression coefficient of optical density on total pigment (in volume per cent O_2 capacity) was 1.286 for 1 cm. light path length; this coefficient was 1.445 when oxyhemoglobin blanks were used. These figures were confirmed for intermediate concentrations in undiluted hemolyzed blood in several cases by gravimetric combination of solutions from oxyhemoglobin and methemoglobin cells (i.e., treated with $NaNO_2$ and washed).

The pH of undiluted hemoglobin solutions obtained by freezing whole blood varied only slightly, and lay between 7.2 and 7.3.

DISCUSSION

Previous spectrophotometric techniques[1-12] have used blood more or less altered by dilution or the addition of hemolytic agents. In our experience these methods have proved unsatisfactory. Errors of measuring the small quantities

of whole blood and saponin solution usually exceed considerably the reading error of the spectrophotometers. The dark brown saponin solutions become cloudy in less than one day and they frequently produce an increasing cloudiness in the blood after the two are combined and during the readings. Several detergents and other hemolytic agents were tried and found to be no better, or to require larger volumes. The effect of the oxygen tension of an added solution is probably also variable. Drabkin and Singer[19] attempted to use unhemolyzed blood in a very thin cell; their equations deal with the extreme turbidity of whole blood in terms of a function of the red cell count. Our own experience agrees with the older observation[3] that unhemolyzed blood yields more variable results, even when a portion of the same blood is used as a blank. Similar conclusions may be drawn from recent modifications.[13] A major portion of the optical density is due to some other difference between the two cuvettes than that in the pigment. The use of readings taken at 2 wave lengths[10, 12] is subject to the theoretical objection that blood, especially when saponin is added, is clearly not a two-component mixture, and the practical objection that one of the 2 readings is usually taken outside the optimal reading range of the instrument.

Use of a physical method of hemolysis leaves the analysis free of the various errors mentioned. Red cell stroma fragments were shown to have a negligible effect. The quantities of fluoride used in the analyses had no influence on the determination. Use of mineral oil in blood-collecting devices, however, did have a significant and variable effect which was occasionally large. Transillumination of these unusually transparent solutions revealed numerous refractile oil droplets in every instance; use of petrolatum jelly instead of liquid completely eliminated this difficulty. The present data, therefore, were obtained to determine the optical constants of blood in the absence of the previously mentioned variables.

Our results confirm the observations of others, with narrowed variability, that Beer's law applies to this type of analysis through a range of at least 13.28 vol. per cent O_2. A few observations upon the comparison of blood saturated with oxygen and completely reduced by prolonged equilibration with nitrogen-carbon dioxide gas mixtures have confirmed, for this type of hemoglobin-plasma solution, the application of Beer's law to a range of 21.30 vol. per cent difference (complete absence of oxyhemoglobin from the test cuvette). The interindividual constancy of optical properties of hemoglobin-oxyhemoglobin mixtures is shown, within the limits of error of manometric oxyhemoglobin determination. The molar extinction coefficient difference of 0.722, however, is significantly different from that previously reported, 0.695,[11] at 660 mμ, using saponin and dilution.

The standard error of estimate of 0.03 in the present method corresponds to a range of variability of 0.18 vol. per cent for difference between 2 samples for fiducial limits of 2σ, and is within the limits of variance of the manometric determination of A-V difference. Values of $\sigma = 1.9$ per cent saturation (i.e., $2\sigma =$ about 0.76 vol. per cent),[10] $\sigma = 1.5$ per cent (i.e., $2\sigma = 0.60$ vol. per cent),[12] $2\sigma = 0.46$ vol. per cent[11] (our value for this method, $2\sigma = 0.40$ vol. per cent), and $2\sigma = 0.66$ to 0.80 vol. per cent[13] have previously been reported.

The molar extinction coefficient, at 540 mμ wave length, of cyanmethemoglobin obtained from the frozen bloods was 11.60 (σ = 0.09) by comparison with Drabkin's value of 11.5,[16] the error said to be 0.5 per cent,[9] and Meyers' value of 11.75, σ = 0.23.[1] The method of correction for dissolved O_2 is not known for the latter data. These values therefore seem to be essentially unchanged by the use of frozen blood.

Accurate analysis of blood in solutions of this type for other pigments than reduced hemoglobin suggests itself. The application to methemoglobin analysis mentioned serves as an illustration. Such minute quantities cannot be measured by dilution techniques, but require both a highly concentrated solution and one which is free of dilution and other errors. Although the light absorption of methemoglobin is affected by pH of the solution at most wave lengths, at 635 mμ there is little effect of pH between 7.07 and 7.48.[20] The pH of the freeze-thaw solutions used is largely determined by the balance between plasma bicarbonate and intracellular fixed acids, the free CO_2 being reduced by aeration. The accuracy of such a method for methemoglobin depends in part on the constancy of these two buffers under most circumstances.

SUMMARY

1. A method is described for blood O_2 analysis which is rapid and simple and is free of errors of dilution, contamination, and particulate material, since complete hemolysis is effected by freezing and thawing.

2. Studies by this method of the optical constants of bloods of varying oxyhemoglobin content suggest that these properties do not vary significantly from one human subject to another and that spectrophotometric analysis of blood for O_2 content should be possible with a reliability at least equal to that of gasometric methods.

3. The molar extinction coefficient difference of uncontaminated oxy- and reduced hemoglobin in blood was found to be 0.722 O. D./mM/L./cm. light path at a wave length of 660 mμ, a higher value than that reported in the presence of saponin.

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AN ELECTRONIC SWITCH FOR MONITORING TWO PHYSIOLOGIC EVENTS SIMULTANEOUSLY ON A SINGLE-BEAM OSCILLOSCOPE

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INTRODUCTION

IT IS frequently desirable to monitor the curves of two physiologic variables such as blood pressures and electrocardiographic potentials continuously and simultaneously throughout a surgical procedure. The usual device for this purpose is a cathode ray oscilloscope having two separate beams. These oscilloscopes are expensive, bulky, and rather difficult to operate because of the excessive number of controls. The device to be described is an electronic switch which will produce two separate curves on a single-beam oscilloscope. The single-beam oscilloscope is much less expensive, more commonly available, smaller, and simpler to operate.

MATERIALS AND METHODS

The operation of the electronic switch is as follows: Two separate signals, A and B, are connected to the two inputs of the switch, and the output is connected to a single-beam oscilloscope. The switch positions the oscilloscope beam upward and connects A to the oscilloscope for a brief time. It then positions the beam downward, disconnects A and connects B to the oscilloscope for a brief period. This process repeats indefinitely. Individual gain controls for the two inputs and a control for varying the vertical separation of the traces are available on the front panel of the switch. The switching frequency is very great compared with the heart rate, so that the two traces appear to be simultaneous and continuous.

Up to this point the operation of this switch is similar to that of several commercially available units. To the knowledge of the writers, all commercial switches are of the single-ended or unbalanced type. In the usual case that the physiologic signal source is a push-pull or balanced type, the use of the ordinary commercial switch is undesirable for the following reasons: (1) Any hum or noise appearing in phase at the two sides of a push-pull amplifier will cancel in the oscilloscope if the amplifier is connected by means of a push-pull switch to both deflection plates of the oscilloscope. This will not be true if a single-ended switch is used, so that the interference level will be higher. (2) In push-pull circuits the push-pull signal carries the desired information. There are cases where one side of the signal is not of the same shape as the push-pull signal. Here, a single-ended switch would cause errors. Accordingly, a push-pull or balanced electronic switch was designed having a switching rate and circuitry appropriate to the type of signals and equipment in common use for physiologic research.

Since resolution is limited by the oscilloscope spot diameter at the operating trace velocity, little additional information is obtained by switching at a rate faster than that which will produce a center-to-center blank interval equal to the spot diameter. This switching rate is determined by the maximum spot velocity, which will occur during the rise of the R

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wave of the electrocardiogram. Here, a spot 1 mm. in diameter must move 15 mm. in 0.02 second, or 750 mm. per second. Since one switching cycle occupies a distance of 2 mm., the required switching frequency is 375 cycles per second.

If this frequency is used, the R-wave rise will resemble a string of beads. If this beading effect is not desired, it will be necessary to increase the switching frequency. Defining beading as the percentage variation in the width of the trace with respect to the spot diameter, it can be shown that beading can be reduced to 14 per cent by doubling the minimum rate given previously. In practice, the quality of the switching action deteriorates at higher rates, and since little additional information is obtained, the rate of 375 cycles per second was chosen.

The frequency response of the amplifiers in the switch must be uniform from less than 1 cycle per second to a high limit set by the resolution of the oscilloscope screen. This is certainly not far above 750 cycles per second, as concluded from the previous discussion. Theoretically, the amplifier response should decrease above this frequency to reduce noise. However, there appears to be no simple way to reduce the amplifier band width without deteriorating the switching action. Accordingly, the amplifiers were left with response to much higher frequency. The noise level is perfectly satisfactory.

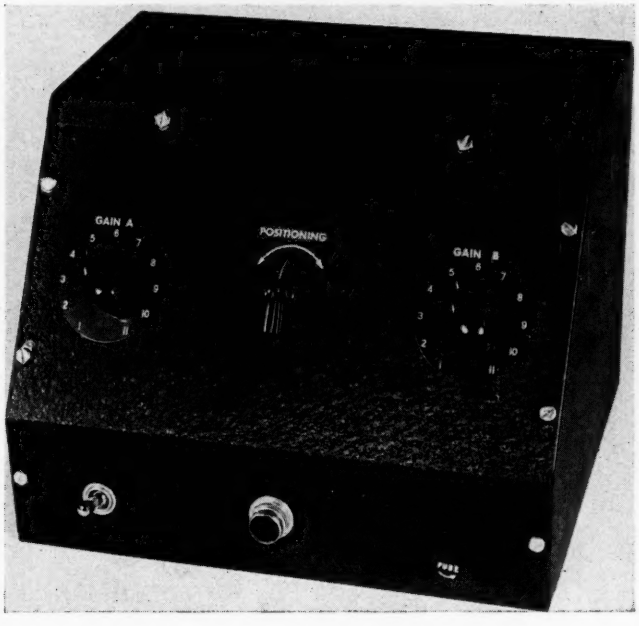

Fig. 1.—Frontal photograph of the electronic switch showing the simplicity of the controls.

The two inputs to the switch are obtained from separate single-ended or push-pull sources having at least 2.5 volts peak-to-peak excursion. Such a source might be the terminals at the recording pen of a direct-writing recorder such as the Sanborn Polyviso. The output of the switch connects directly to the vertical deflection plates of a single-beam oscilloscope such as a DuMont 304-A. The sweep time is slowed to approximately four seconds by the use of additional capacitance in the timing circuit of the sweep generator. Type P-7 phosphor is required on the cathode ray tube.

The switch is shown in Fig. 1. The center knob controls the vertical separation of signals A and B on the oscilloscope screen. By manipulation of this knob, A and B may be superimposed or separated any desired distance, with either A or B on top. Regardless

of their separation, the two traces will always be symmetrically disposed with respect to a horizontal line through the center of the tube face. The other two knobs are separate gain controls for the two inputs.

Fig. 2 is a view of the switch showing the mechanical layout.

The schematic diagram of the switch is given in Fig. 3. The 12AT7 tube is a free-running multivibrator which switches the 6BE6 amplifiers on and off in pairs. Thus, for one regime of the multivibrator, A and A′ are amplifying while B and B′ are not. When the other regime of the multivibrator occurs, A and A′ are not amplifying, while B and B′ are. The process repeats indefinitely. Tubes A and A′ comprise a gated push-pull amplifier for input A, while tubes B and B′ comprise a similar amplifier for input B. The plates of A and B are paralleled, as are the plates of A′ and B′. Now since only one of the paralleled plates is conducting at any time, the plate potential of each pair is controlled by the screen voltage of the tube which is conducting at the time, as well as the signal voltage on its grid. For proper operation of the dual potentiometer position control, it

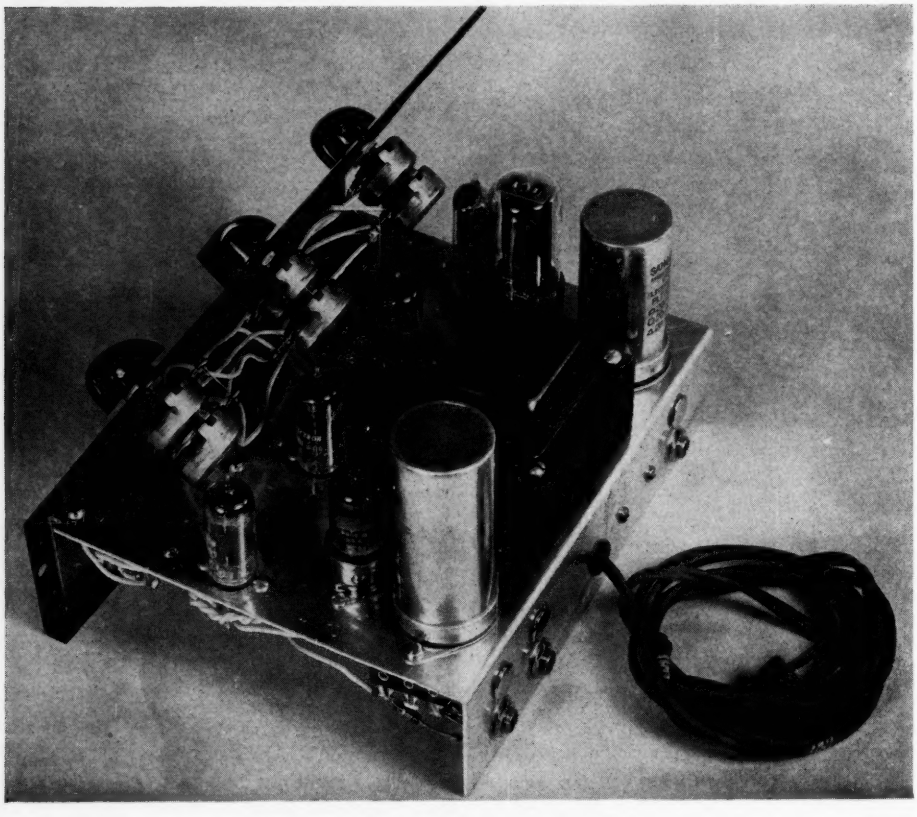

Fig. 2.—View of the electronic switch showing the mechanical layout of the electronic components.

must be connected so as to increase the resistance in the screen of A and decrease the resistance in the screen of A′ as the shaft is rotated. The dual gain potentiometers are connected so as to increase the signals on both grids simultaneously as the shaft is rotated. Note that the input and output jack connections are inverted to correct for the signal inversion in the amplifiers.

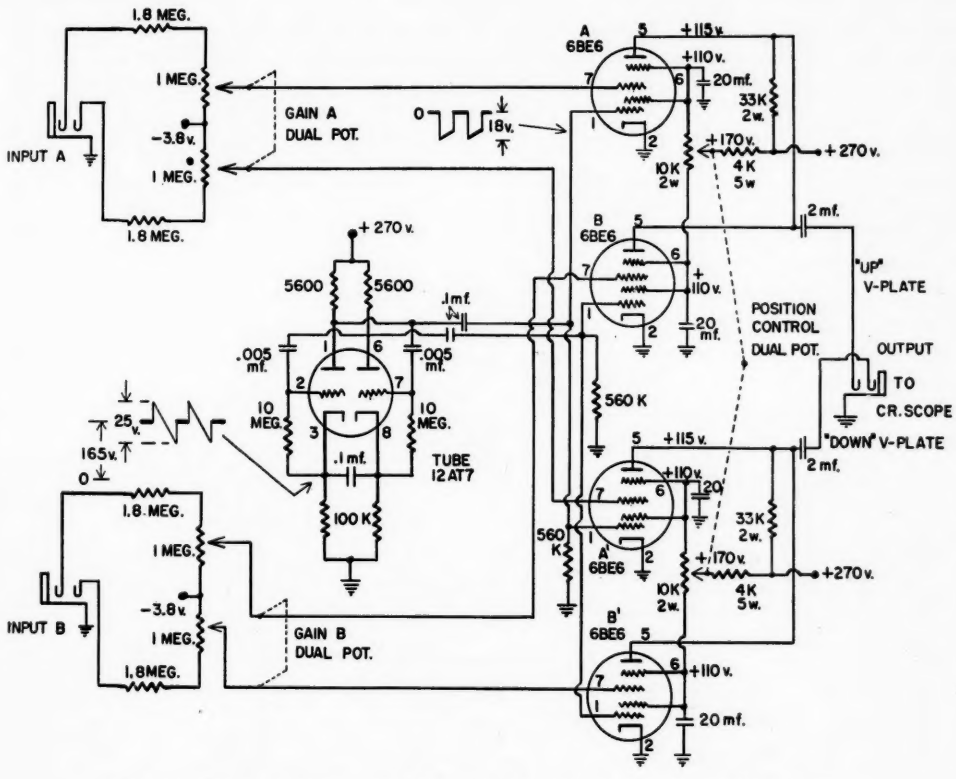

Fig. 3.—Electrical schematic diagram for the electronic switch proper.

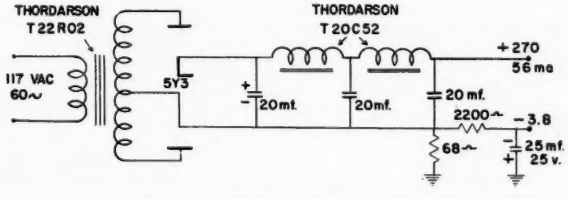

Fig. 4.—Electrical schematic diagram for the power supply section. The components of both Fig. 3 and Fig. 4 are contained in a single cabinet.

As illustrated in Fig. 4, the power supply for the switch is conventional except for the employment of "back bias" to obtain fixed bias voltage for the 6BE6 amplifiers. The entire switch, complete with its power supply, is contained in a sloping front metal cabinet measuring approximately 7 by 7 by 9 inches (Bud C-1585).

Fig. 5, *A* illustrates the method for connecting the output of the Sanborn Polyviso to the switch input. Attachment to the 6V6 output cathode followers may be made directly at the pen or at the terminal strip at the rear of the rack. The 330K resistors and 0.01 capacitors are low-pass filters to reduce noise without materially deteriorating the desired signals. The 0.5 capacitor is simply a coupling unit to prevent the large direct voltage component at the pen from being applied to the switch input. All of the components shown outside of the dashed line may be placed in a small metal box and secured to the Polyviso frame at any convenient point. Clearly, two such units are required.

Fig. 5, *B* illustrates the method of connecting the output of the switch to the DuMont 304-A oscilloscope. The wire jumpers shown as dashed lines are removed and a jack is wired as shown. The jack may be mounted on the terminal board. With this connection, the horizontal and vertical position controls on the oscilloscope are operative, and the sweep circuit

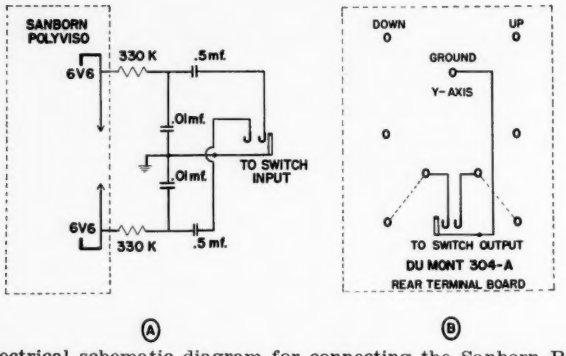

Fig. 5.—*A*, Electrical schematic diagram for connecting the Sanborn Polyviso to the electronic switch; *B*, electrical schematic diagram for connecting the electronic switch to the DuMont 304-A oscilloscope.

operates normally. The y-axis amplifier does not amplify the signal voltage, but is used merely to provide position control and an appropriate positive deflection plate potential to prevent defocusing the beam.

RESULTS

Performance data are as follows:

Position control: More than full-screen height.
Sensitivity: 0.9 volt RMS per peak-to-peak inch.
Gain: Fourteen times.
Frequency response: Uniform from D.C. to over 750 cycles per second.
Maximum input voltage: 3 volts R.M.S.
Linearity: Excellent 0-3 volts R.M.S. input (3-inch peak-to-peak deflection).
Cross-talk: With 3 volts input to *A*, zero input to *B*, position at balance, 2 per cent. Proportionately less cross-talk within normal operating range.

The switch described in this article has been in almost daily use in our cardiac catheterization laboratory for over six months and has been very satisfactory from the clinical point of view.

SUMMARY

A simple, inexpensive electronic switch using only common radio components is described, together with adapting circuits to permit simultaneous two-channel monitoring on an ordinary single-beam oscilloscope. The switch is dependable, accurate, and simple to operate and maintain.